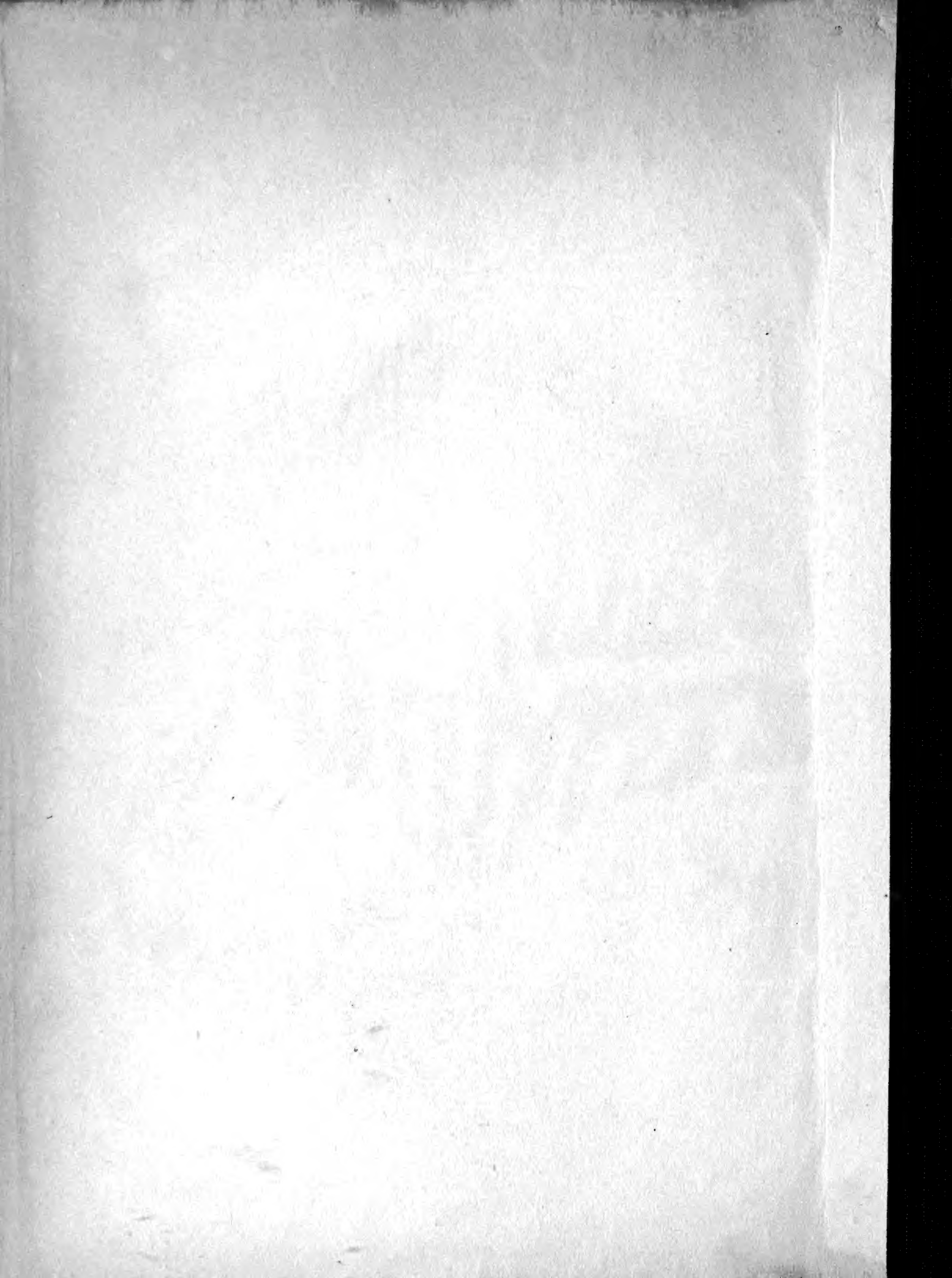




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BACTERIA IN RELATION TO PLANT DISEASES.









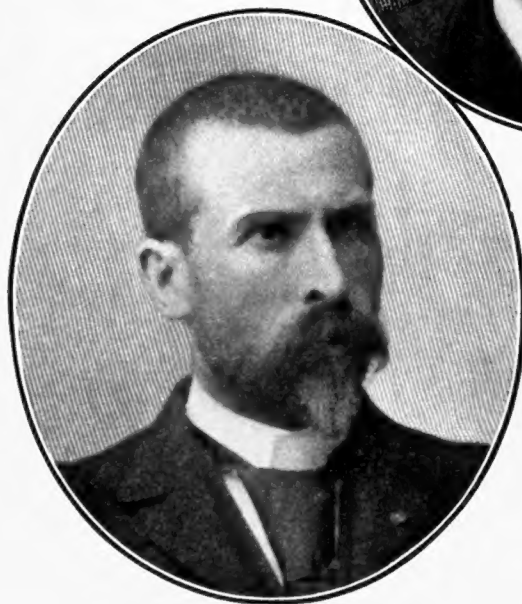
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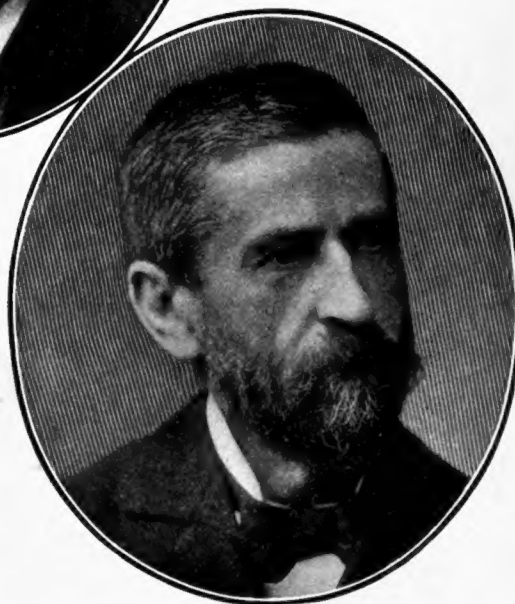
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1. FERDINAND COHN.

2. ROBERT KOCH.

4. ÉMILE ROUX.

3. LOUIS PASTEUR.

5. ÉMILE DUCLAUX.

Bot.  
Gen.  
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# BACTERIA IN RELATION TO PLANT DISEASES

BY

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VOLUME ONE.

METHODS OF WORK AND GENERAL LITERATURE OF BACTERIOLOGY  
EXCLUSIVE OF PLANT DISEASES.



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## PREFACE.

The subject of bacterial diseases of plants is older than the poured-plate method of Koch, but until recently our knowledge of it has been in a very chaotic state, it having been for the most part for twenty-five years a recognized but uncultivated field. In recent years, however, publications on plant bacteria have multiplied, and they now amount to several hundred titles.

The writer's studies of the bacteria themselves and of the diseases which they cause, as distinct from the literature of the subject, began in 1893. At that time there was very little reliable information on this subject. The literature is now more extensive, but it is nowhere gathered together in one place and properly summarized. It has seemed, therefore, for a long time, that a work of the scope of the treatise here presented might be clarifying and useful to many people. There have been published, and are still appearing, so many papers on the subject of bacterial diseases of plants by writers ignorant of bacteriological methods and indifferent to the requirements of modern pathological inquiry that this whole subject has been brought into disrepute. This is the only possible explanation of the fact that up to a very recent date writers on pathology and bacteriology have been telling their readers that there is no good evidence of the existence of any such diseases.

The following editorial paragraph from the *Botanical Gazette*, February, 1893, may be cited as indicating the general feeling on this subject at that date:

What is especially needed at this stage of advancement is the continuous and systematic examination of the whole ground by one or more well-equipped investigators, and the publication of a critical statement of what may be safely accepted as proven. Even a summarization of the present status of the subject, without critical laboratory study, would be helpful, if well done.

That this feeling has become intensified with the progress of time and the multiplication of literature is shown by the following citation from the large *Treatise on Bacteriology*, by Miquel and Cambier, published in 1902:

The list of bacteria capable of attacking the higher plants increases rapidly from day to day; but whether the experiments of plant pathology offer greater difficulties than those of animal pathology, or whether the authors who have undertaken them have ignored the multiple resources which bacteriology offers to-day, many of the species described must be studied anew, their monography offering regrettable lacunæ. By the side of some fruitful and well-conducted labors we find, unfortunately, altogether too many which must be done over entirely.

It was with the hope of making useful discoveries and clearing up part of this contradiction and uncertainty that the writer began his study of this class of diseases. His first effort in the way of preparation was to supplement his botanical training with a knowledge of bacteriological methods which he obtained from standard literature and competent teachers. His second effort was to gather

together and properly digest all of the literature relating to this subject. This resulted in the projection of a critical review of the literature, begun in 1896 in the *American Naturalist* but left unfinished, owing to pressure of research work, and a card catalogue which is now here published in full with critical remarks. His third endeavor was to carefully work over, in the laboratory, field, and greenhouse, as opportunity offered, all of the so-called bacterial diseases of plants, submitting each supposed parasite to all of the tests of modern pathology. The latter has proved a far larger undertaking than was anticipated, the number of diseases attributed to bacteria having increased rapidly in recent years. It is expected that more than 125 diseases will be treated or touched upon in this monograph, many of which have come under the writer's own observation. An attempt has been made to cover the literature of the whole world and to work over personally every disease so far as material could be obtained.

The present volume contains an "outline of methods of work," which was written up in substantially the same form four years ago, in connection with the investigations of the Laboratory of Plant Pathology, Bureau of Plant Industry, United States Department of Agriculture, its publication having been delayed in order to bring the rest of the manuscript into suitable shape. The monograph is published in this form with the approval of the Secretary of Agriculture.

The bibliography at the end of this volume covers the general subject of bacteriology, exclusive of plant diseases, and is arranged chronologically by subjects. Not every good paper is referred to, but for the most part only such as have fallen under the writer's own observation. It is believed, however, that by consulting these the student will soon be able to get hold of the entire literature of any special branch. The reader who wishes to keep pace with the advance of the science should consult the International Catalogue (R) published by the Royal Society of London.

The illustrations, especially those dealing with histology, have been drawn, with very few exceptions, under the direct personal supervision of the writer, every one of them when near completion having been inspected critically and modified in various details to correspond as closely as possible to the actual object. The slides from which the drawings have been made will be placed on file in the Laboratory of Plant Pathology, where they may be consulted.

This monograph is not intended to take the place of ordinary text-books of bacteriology, of which there are now many, but rather to supplement the same, giving information where they are silent or misleading. It is hoped that it will be of value not only to plant pathologists, for whom it is primarily intended, but also to physicians and animal pathologists for purposes of comparison. In its preparation the writer has had also an eye to the service of gardeners, fruit-growers, and all who take an intelligent interest in plants. It is presented with a keen sense of its imperfections, but with the hope that it may at least serve its main purpose. While the writer has made every effort to be accurate in statement and just in criticism, it is too much to hope that he has always succeeded, and, therefore, he desires to crave pardon in advance for all errors of omission and commission, taking



shelter behind Lavoisier's well-known and convenient apology: "Man would never give anything to the public if he waited till he had reached the goal of his undertaking, which is ever appearing close at hand and yet ever slipping farther and farther as he draws nearer." Those who dwell in the clearer light of the next generation will build better than we have done and will scarcely realize how slowly and painfully many of us have groped about for what seems to them so plain.

In conclusion, I desire to make grateful mention of Dr. Theobald Smith, professor of comparative pathology in Harvard University Medical School, and Dr. Veranus A. Moore, professor of comparative pathology and bacteriology in Cornell University, each in turn in charge of the animal pathological investigations of the Bureau of Animal Industry, United States Department of Agriculture, at a time when the writer was beginning his bacteriological studies and was perplexed in many ways. To friendly advice and helpful suggestions from these distinguished men he owes more than he can well repay.

AUGUST 25, 1905.



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BACTERIA IN RELATION TO PLANT DISEASES.

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BY ERWIN F. SMITH.

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# BACTERIA IN RELATION TO PLANT DISEASES.

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## PART I.—AN OUTLINE OF METHODS OF WORK.

### GENERAL REMARKS.

The following outline of methods for the study of bacterial diseases of plants, which are now in use in the Laboratory of Plant Pathology, United States Department of Agriculture, has gradually assumed its present shape as a result of the writer's field, hot-house, and laboratory experiments during the past thirteen years. In nearly the same shape, so far as arrangement is concerned, but in a less complete form, it was published in the *American Naturalist* in 1896.\*

The scheme here presented is entirely practicable and is believed to be not more extended than the exigencies of the case require; in the interest of better methods of work in plant pathology it is recommended to all who contemplate a special study of bacterial diseases of plants, and also particularly to those who intend to describe and name species of bacteria, whether pathogenic or nonpathogenic. Those who doubt the necessity for so much work are advised to read procedures recommended for the study of bacteria by a committee of the American Public Health Association, and the earlier paper by H. Marshall Ward (*Bibliog.*, III).† It would be still more to the point if they would isolate a dozen bacterial organisms from the soil, air, or water, and undertake faithfully to identify them by means of any of the older descriptive works, *e. g.*, Eisenberg's *Diagnostik* or Saccardo's *Sylloge Fungorum*, or even by such recent manuals as those of Sternberg, Lehmann & Neumann, Flügge, Migula, or Chester (*Bibliog.*, III). Everyone who has carefully inquired into the matter knows that the brief statement of the behavior of an organism on nutrient agar, on gelatin, and on two or three other media, with perhaps a loose statement of its color and size, no longer constitutes a description which describes. Such accounts, of which there are a great many, usually fail to mention just those things which might serve to distinguish the organism from its fellows. If a new species is not to be described so that it can be identified by others, what then is the use of any name or any description? The name will only serve to encumber future synonymy and to recall the incapacity of its author.

\*The bacterial diseases of plants: A critical review of the present state of our knowledge, parts I-vi, *Am. Nat.*, August and September, 1896.

†For Bibliography see end of volume.

## THE DISEASE.

The line between disease and health is sometimes a very narrow one, especially when nothing more is involved than some slight change in function. The difference, however, is very striking in many of the diseases here considered. The writer has used the word "disease" in the common acceptance of the term, meaning thereby

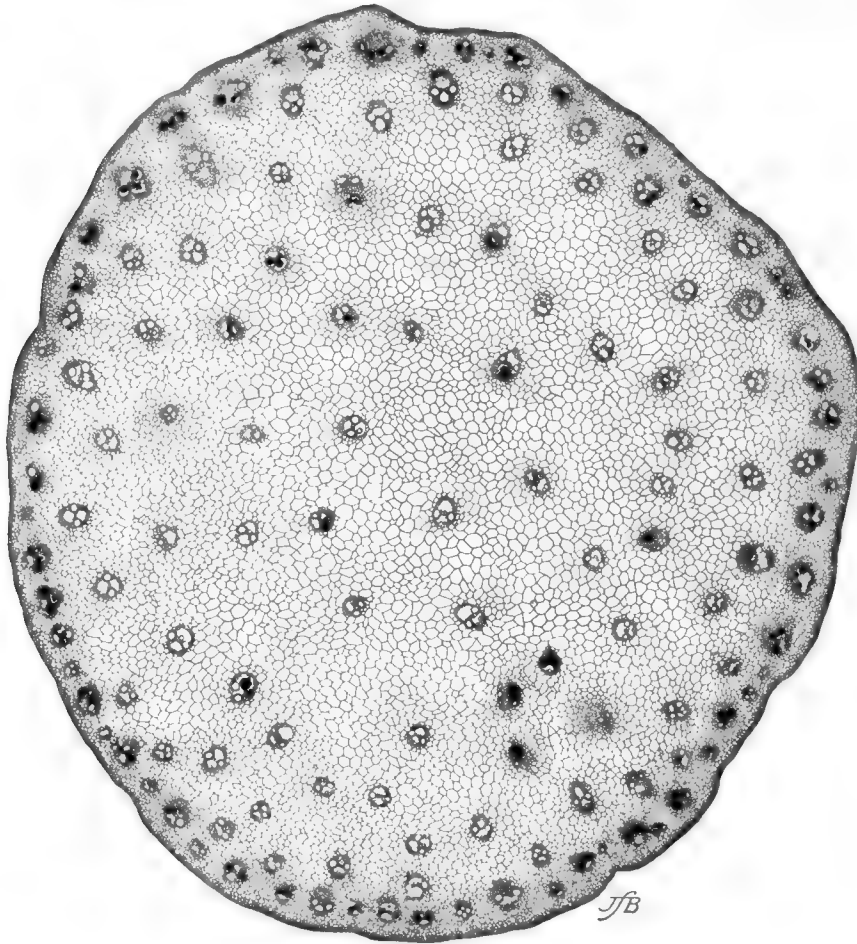


Fig. 1.\*

any marked deviation from the normal functions or structure of the plant as it now exists, whether wild or greatly modified by cultivation. In a sense, such a change as has taken place in the cauliflower, the normal flower-shoots of which have become

\*FIG. 1.—Cross-section of the upper part of a sweet-corn stem parasitized by *Bacterium Stewarti* (Erw. Sm.). The location of the bacteria is indicated by black shading. Most of the affected bundles are on the periphery. The bacteria have not escaped into the parenchyma. Jamaica, Long Island, N. Y., July 16, 1902. The section was taken several feet from the ground, but the stem infection undoubtedly took place through one or more of the lower nodes. Drawn from photomicrograph of a section stained with carbol-fuchsin. Exactly similar sections, but with a larger number of infected bundles, have been cut from stems of sweet-corn plants infected by the writer in August, 1902, during the seedling stage shown in fig. 73.

compacted, aborted, and enlarged into a fleshy edible mass, might well be regarded as a diseased condition, but it is not so regarded for the purposes of this book. On the contrary, a soft rot of the cauliflower head is regarded as a disease. Bacterial diseases of plants usually involve both functional and structural changes.

Inasmuch as the word "symptoms" has a subjective as well as an objective connotation in medical terminology, the writer has preferred to substitute the word "signs" for those objective characters which serve to distinguish one plant disease from another.

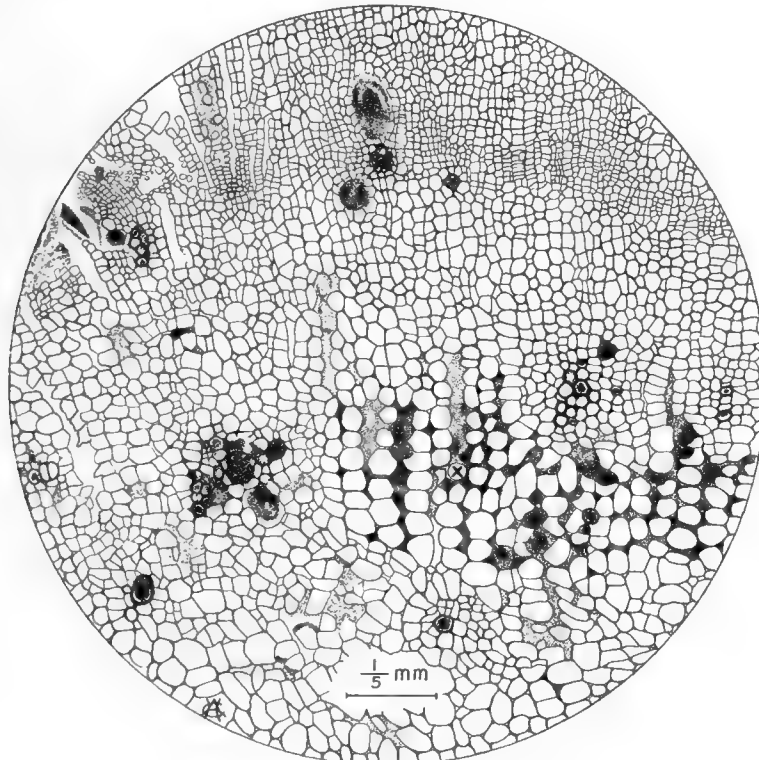


Fig. 2.\*

The student will, naturally, first turn his attention to a careful study of the disease. Under this head should be considered: (1) Previous literature; (2) Geographical distribution; (3) Signs of the disease; (4) Pathological histology; (5) Direct-infection experiments.

\* FIG. 2.—Cross-section of a raw carrot, showing wedging apart of parenchyma cells by *Bacillus carotovorus* Jones; from paraffin-infiltrated material. The carrot was fixed in strong alcohol 72 hours after placing on its cut surface one loop of a fluid culture. The inoculation was made in the middle of a cross-section of the whole root, 1 cm. thick, placed in a sterile Petri dish. The surface of the root was sterilized in mercuric chloride water. This section was made several millimeters below the inoculated surface. A small portion of it at X is shown more highly magnified in fig. 3. This section was stained with carbol-fuchsin and bleached in 50 per cent alcohol. Drawn under Zeiss 16 mm. apochromatic objective with No. 4 compensating ocular and the Abbe camera.

In the present state of our knowledge (1) and (2) can usually be considered only after a very careful study of (3), (4), and (5), and of the organism itself. They involve a knowledge of modern languages, and a very considerable familiarity with scientific literature.

#### PREVIOUS LITERATURE.

One of the first requisites in a student is a knowledge of how to use literature. Previous literature is, however, often of such a fragmentary and uncertain sort, as we shall see, that it is impossible to decide whether a disease is actually new or has been written upon before.

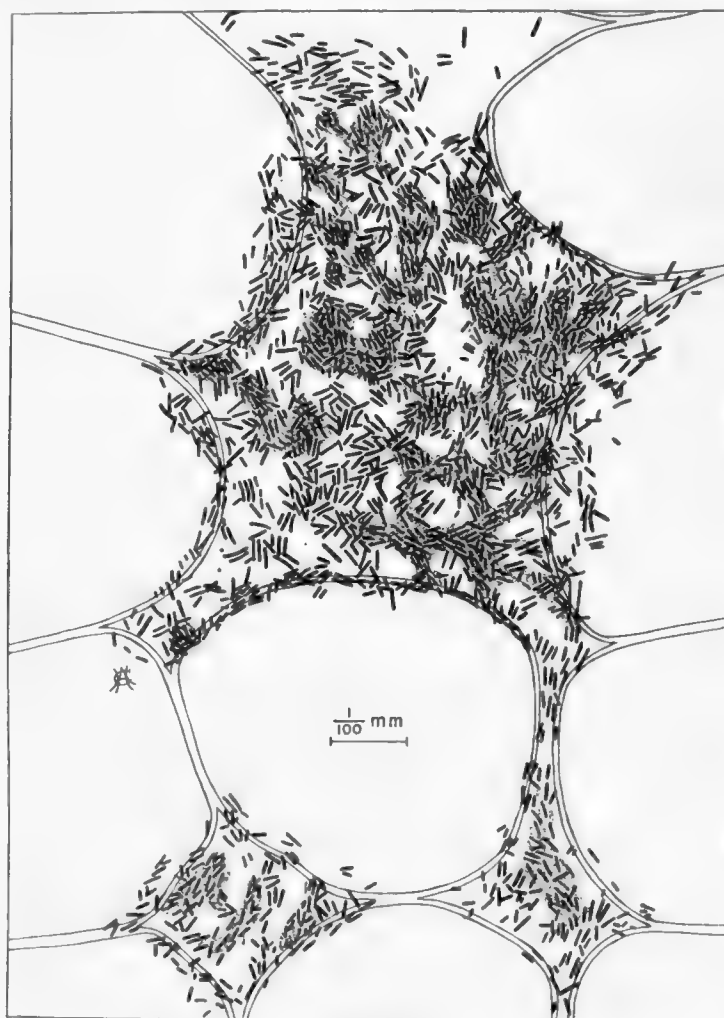


Fig. 3.\*

The literature of plant diseases will not be referred to in this volume, except occasionally and incidentally. The bibliography of this volume deals only with general bacteriology—human and animal diseases, methods of work, etc.

\*FIG. 3.—A detail from fig. 2. *Bacillus carotovorus* wedging apart cells of the carrot. Drawn mostly from one plane. In placing the cover-glass a few of the bacteria have been crowded out of the intercellular spaces into parts they did not originally occupy.  $\times 1,000$ .

## GEOGRAPHICAL DISTRIBUTION.

Geographical distribution is an exceedingly interesting problem to many naturalists. The writer shares this feeling and has made every effort to determine it, as far as possible, for each disease. There are, however, still many gaps in our knowledge—the whole subject is so new, and information from all parts of the world is desired. The inner temperature of plants conforms nearly or quite to that

of the surrounding medium, and we might therefore expect, in some cases at least, to find a rather more sharply restricted distribution than in diseases of the warm-blooded animals. From theoretical considerations we should expect the distribution of plant diseases to be more like that of diseases of fish and other cold-blooded animals. Whenever the bacterium is able to endure as wide a range of temperature as the host-plant, we should expect to find it as widely distributed.

## SIGNS OF THE DISEASE.

Great care should be exercised in the description of the physical signs and of the lesions due to the parasite, so that the disease may be identified from these alone, if necessary. A great many cases should be examined and the signs must be recorded in detail and with great accuracy. It should be remembered that here is a frequent opportunity for error to creep in, since the plant may be affected by

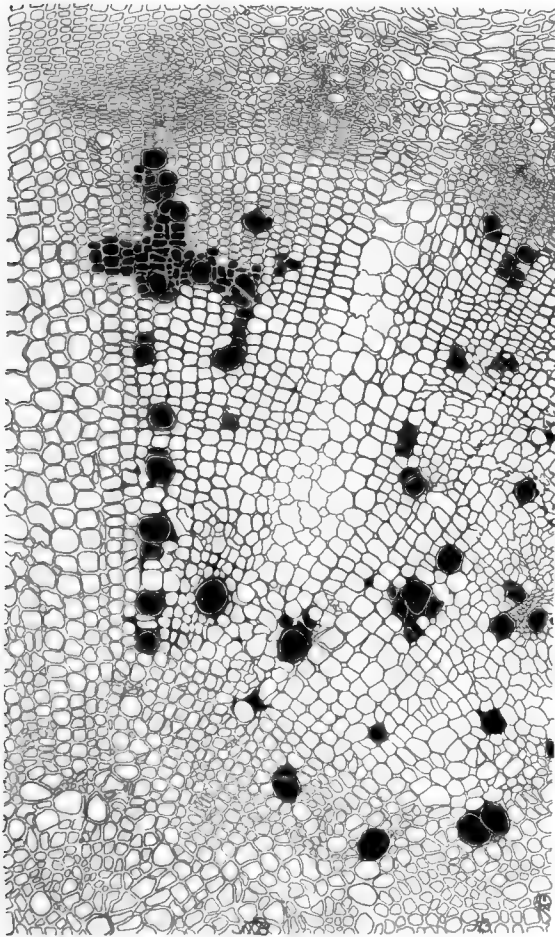


Fig. 4.\*

two distinct diseases which have been confused. Good figures are always desirable, but are not absolutely essential. If possible, however, photographs, pen or pencil drawings, and good water-color sketches should be secured.

\*FIG. 4.—Cross-section of a turnip root, showing vessels occupied by *Bacterium campestre* as the result of a pure-culture inoculation by means of needle-pricks on the leaves. Material fixed in strong alcohol, infiltrated with paraffin, cut on the microtome, stained with safranin-picro-nigrosin, and the differential washing stopped at just the right stage. The bacteria are confined to the vessels and their immediate vicinity. They do not occur in the phloem, a small portion of which is shown at the top of the picture. Section made from the same root as fig. 6, but lower, in the tapering part. Drawn from a photomicrograph.  $\times 85$ .

When all is said, the signs of many plant diseases, it must be admitted, are much alike, and this is particularly true of the bacterial soft rots. This is an added reason for studying them in each case as critically as possible. The captious reader might also remember that while an enormous amount of painstaking labor has been devoted to animal pathology, including twenty centuries in case of human medicine, we are only in the beginning, so to speak, of our knowledge of the minute pathology of plant diseases, and especially of those due to bacteria.

#### PATHOLOGICAL HISTOLOGY.

The relation of the parasite to the tissues of the host should be studied both in fresh material and in stained microtome sections made from material properly fixed and infiltrated with paraffin. The organism may be a wound-parasite, or it may be able to enter through uninjured parts, *i. e.*, in the absence of visible wounds. Often it affects special tissues or systems of tissues. Sometimes the bacteria are quite closely restricted to the vascular system, forming occlusions (figs. 1, 4, 5, 7, and 9). Sometimes they spread widely in the intercellular spaces of the parenchyma, forming numerous cavities (figs. 2, 3, and 6). Sometimes there are striking reactions on the part of the host, *e. g.*, an enormous multiplication of cells resulting in cankers or tumors (plate 2). The habits of the parasite and the behavior of the tissues of the host are best learned from serial sections. The student should not fail to preserve (properly labeled) in strong alcohol an abundance of typical diseased material for future study, exchange, or reference. Stained cover-glass preparations and stained sections should also be mounted in xylol-balsam, carefully labeled, and filed away. Neglect of these precautions prevents the experimenter from furnishing the convincing proofs in case his printed or oral statements are called in question.

As to the best methods of *fixing* plant material containing bacteria much remains to be learned. The writer has had best success with strong alcohol (90 per cent to absolute) and with picric acid dissolved to saturation in absolute alcohol and used boiling hot. In general the watery fixatives can not be used because they do not hold the bacteria in place; even alcohol as strong as 70 per cent allows many kinds of bacteria to diffuse out into the fluid. Boiling absolute alcohol saturated with mercuric chloride is sometimes useful. The alcohol may be boiled in an open Erlenmeyer flask set on wire gauze on an iron tripod over a small Bunsen flame. The alcohol is first brought to a boil. The pieces of tissue are then thrown in and allowed to remain 3 to 5 minutes. It is better to divide the material into pieces suitable for embedding before fixing rather than after. Usually such a piece should not measure more than one-half square centimeter or one-half cubic centimeter. As far as possible only fresh material should be used for this purpose. Old material has often absorbed air in quantity sufficient to render infiltration with paraffin impossible or at least very difficult. In such cases infiltration in vacuo will often render good service. The writer uses a specially devised air-tight paraffin bath connected to the vacuum-pump. Even this device will not in every instance insure perfect infiltration.

## DIRECT-INFECTION EXPERIMENTS.

Direct-infection experiments will frequently separate out a parasite which is overwhelmed by some saprophyte and thus furnish better material for plate-cultures, and they are also sometimes very useful when one is remote from laboratories and so situated that it is impossible to obtain pure cultures. It is, however, a crude method and only to be employed when more exact methods can not be used or would not serve as well. By "direct" infection is meant the transfer of fluids or solids from the diseased plant directly into the tissues of the healthy plant, an effort being made to include some of the supposed parasites in this transfer. It is a convenient expression and will be used often in this book.

## THE ORGANISM.

This may be considered under three heads—its ability to produce disease, its form, and its physiological peculiarities. Many of the latter might equally well be denominated cultural characters, and the pathogenic properties really belong under physiology, but are kept distinct for sake of convenience and because they constitute not only the most important attributes of the organism, economically speaking, but also a distinct and peculiar phase of the investigation.

## PATHOGENESIS.

What constitutes proof of the pathogenic nature of any organism? Upon the ability of the student to give a proper answer to this question depends very largely his success or failure as an investigator. Henle perceived clearly what was necessary as long ago as 1840, and Koch's rules are still fresh in the minds of all. There is consequently now so good an understanding of this subject among animal pathologists and professional bacteriologists that if this book were designed principally for such persons no comment would be necessary. A glance, however, at the literature of plant diseases shows that many of the writers on bacterial diseases of plants have not had this professional training. The four cardinal requirements, as understood by the writer, are as follows:

## RULES OF PROOF.

- (a) Constant association of the organism with the disease.
- (b) Isolation of the organism from the diseased tissues and careful study of the same in pure cultures on various media.
- (c) Production of the characteristic signs and lesions of the disease by inoculations from pure cultures into healthy plants.
- (d) Discovery of the organism in the inoculated, diseased plants, re-isolation of the same, and growth on various media until it is determined beyond doubt that the bacteria in question are identical with the organism which was inoculated.



Under (a) there should be numerous observations on many plants, with very careful microscopic examination of stained and unstained material. The cells of many plants contain granules which often dance about so actively (pedesis or Brownian movement) as to be very deceptive, and yet they are not bacteria. Living bacteria in plant tissues can always be stained so as to stand out distinctly if the sections are well prepared and sufficiently thin. When bacteria occur in plants as parasites they are usually very abundant in the vascular system, or the parenchyma, or both, and there is, so far as yet known, always a distinct breaking down (solution) of some portion of the tissues (see figs. 6 and 7, and plate 3). If the parenchymatic tissues are sound, if there is no bacterial ooze on making sections, if the vascular

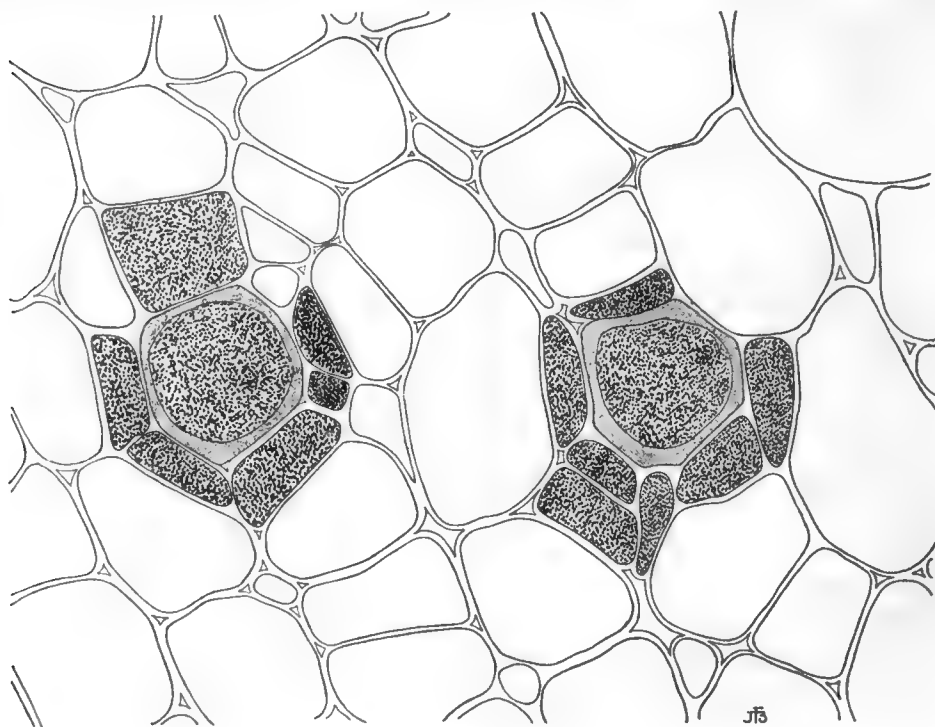


Fig. 5.\*

system is not occupied, and if bacteria can not be demonstrated in the tissues by proper staining, then it is very unsafe to infer their existence from dancing particles, no matter how many may be visible in the unstained sections. Moreover, bacteria may be present in some of the plants and not in others, *i. e.*, not constantly present, and so not the cause of the disease. It is conceivable that they might also be present

\*FIG. 5.—*Bacterium campestre* parasitic in a turnip-root (inoculated plant No. 53). This figure shows the bacteria crowding out into the cells surrounding the reticulated vessels. The lignified portion of each vessel is indicated by fine dots. Material fixed in strong alcohol, infiltrated with paraffin, cut on the microtome, stained with carbol-fuchsin, and the excess of stain removed in dilute alcohol, section then dehydrated and mounted in xylol-balsam. Drawn from a photomicrograph, the contrast here indicated being not greater than that shown in the section.  $\times 500$  circa.





**Bacterial olive-knots produced on four plants by delicate needle-pricks.**

Inoculated January 4, 1904. Photographed May 16, 1904, nearly natural size. The organism came originally from an olive-knot obtained in California, where the disease has been very destructive for a number of years. A pure culture obtained from one of the California knots was inoculated into young growing olive-shoots and numerous knots resulted. From one of these, after about three months, the organism was plated out and a subculture from one of the colonies was used to produce the knots here shown.



quite constantly, but merely as followers of something else. When possible, therefore, diseased plants should be examined for the suspected pathogen, in large numbers, in different years, and from widely separated localities. Of course, if fungi are also present they must likewise be examined as to constant occurrence and pathogenic properties.

Under (b) all of the standard nutrient media should be tried, and that repeatedly, until the student is entirely familiar with the appearance and behavior of the organism. It is usually best to isolate the organism for experiment from selected portions of the tissue by means of Esmarch roll-cultures or by the use of poured plates (Petri-dish cultures), generally the latter.

Isolations may also be made by inserting a sterile platinum needle or loop into the diseased tissue, obtaining therefrom a little fluid, and drawing this over the

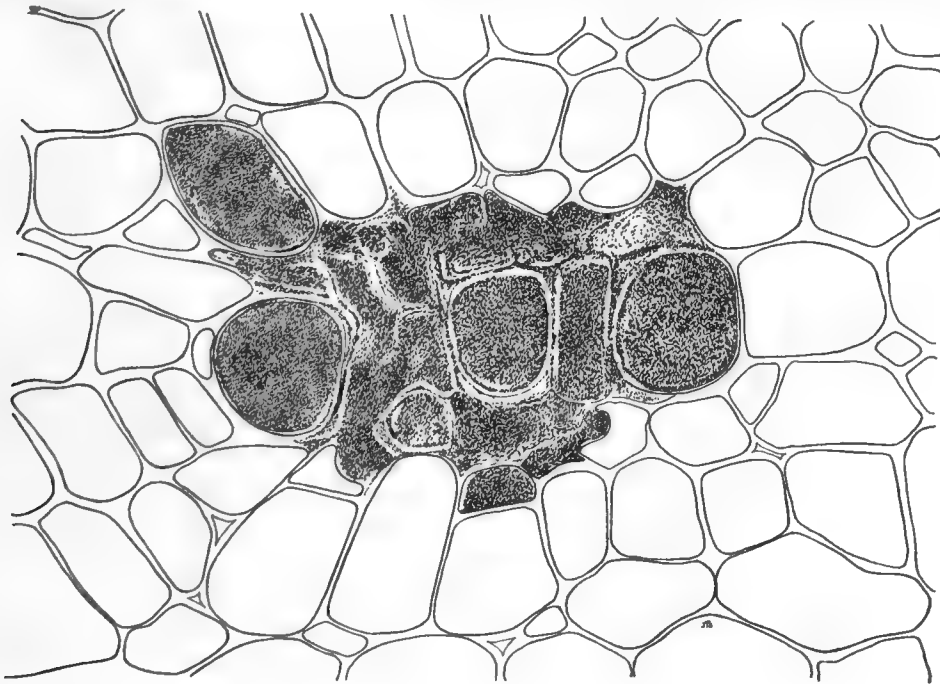


Fig. 6.\*

surface of slant agar, gelatin, or potato a number of times. This is an old method introduced by Koch in 1881. If ten or twelve tubes are used, the final streaks will often consist only of scattering colonies, from one or more of which the subcultures may be made. The plate method has the great advantage of showing just how many kinds of bacteria are present in the tissues (provided they will all grow in the medium used and under the conditions of the experiment), and just how numerous they are. In case of viscid organisms, or those forming compact zooglœæ in the

\*FIG. 6.—Cross-section of root of plant No. 53 (turnip) parasitized by *Bacterium campestre*, showing an early stage in the formation of a bacterial cavity. The original section was made from material fixed in alcohol, infiltrated with paraffin, stained with carbol-fuchsin, and washed in a mixture of alcohol and water. Drawn from a photomicrograph.  $\times 500$ .

tissues, it is sometimes desirable to grow them for a day in bouillon before attempting the plate-cultures; but one must then be on his guard, since it is quite possible by this method to start with enormous numbers of the right organism and have the bouillon culture filled with something else at the end of the 24 hours.

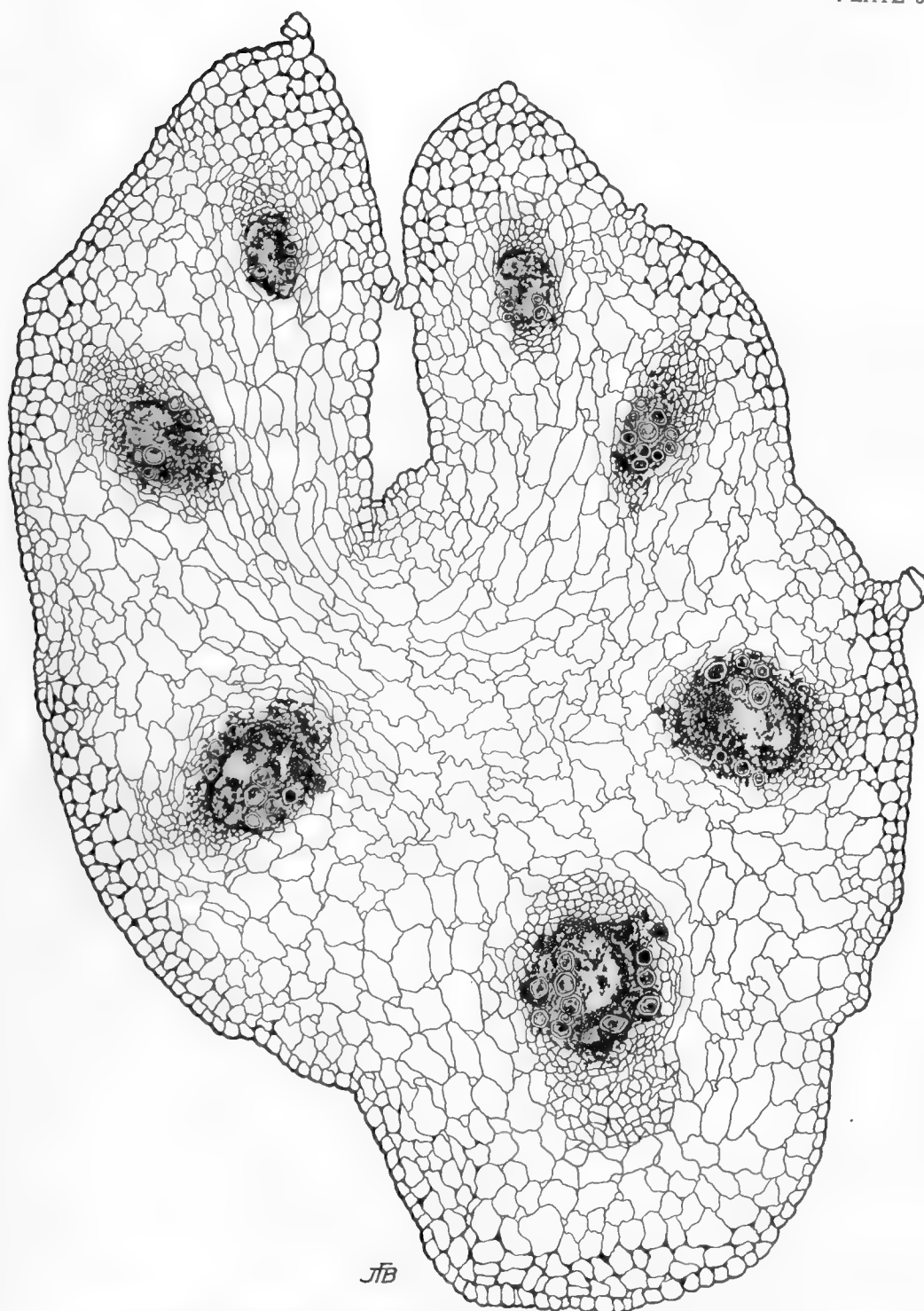
Pure cultures may also sometimes be obtained by cutting out pieces of the tissue and throwing them into tubes of culture media. This method, however, shows little or nothing as to the prevalence of the organism in the tissues, and in



Fig. 7.\*

the hands of beginners is very liable to miscarry. If growth is obtained it may indeed have come from many organisms of one sort pervading the tissues and causing the disease, but it is not certain that it did not result entirely from one or

\*FIG. 7.—Bundle in a cauliflower-petiole entirely destroyed by *Bacterium campestre*. The result of a pure-culture inoculation. Plant No. 112 inoculated March 10, 1897, by needle-punctures on the blade of a leaf without hypodermic injection. First signs of disease March 20. Petiole put into alcohol on April 5. Longitudinal section. Tissues surrounding the bundle entirely free from bacteria. Section not made from the inoculated leaf, but from the first leaf that showed secondary signs. Drawn from photomicrograph of a paraffin section stained with carbol-fuchsin.  $\times 206$ .



Cross-section of petiole of muskmelon No. 150 attacked by *Bacillus tracheiphilus*.

The bacteria are confined to the bundles, in each of which cavities have appeared. This section was taken from near the point marked X on the inoculated leaf (see fig. 8). The inoculations were made on the blade of the leaf by means of delicate needle-pricks. The material was collected and fixed in strong alcohol on the 6th day after the appearance of the disease.



more bacteria accidentally introduced from the surface of the plant, from one's clothing or body, or from the air; or it may have resulted from a few non-pathogenic organisms accidentally present in the inner tissues of the plant, particularly in case of roots which have been dug some time. It is therefore much better for the student to begin with plate cultures. Generally speaking, the parasite will be more easily obtained in a state of purity from plants or organs of plants recently attacked and from deep tissues, or from just within the margin of advancing diseased areas, rather than from near the surface, or from parts which have been diseased for a considerable time.

Parts long affected almost always contain mixed growths due to the multiplication of saprophytes of various kinds. From such parts it is usually much easier to obtain the saprophyte than the parasite, even if the latter has not been entirely crowded out and destroyed.



Fig. 8.\*

Great care must be exercised to avoid introduction of surface organisms which might complicate results, especially if rapid growers. The easiest and most satisfactory way, when the tissues will admit of such treatment, is to sear the surface with a hot knife or spatula so as to burn all surface organisms and then cut or dig through this sterile surface with hot or cold sterile scissors, scalpels, forceps, or needles to a part which has not been affected by the heat, from which some of the diseased fluids and solids may be removed on a sterile platinum loop. I frequently sear upon sound tissues at one side of the spot from which I desire to make cultures

\*FIG. 8.—Muskmelon plant No. 150, inoculated with a pure culture of *Bacillus tracheiphilus*. The pricked leaf is on the left side. The section shown in plate 3 was taken from the point marked X, three days after the photograph was made and ten full days after the inoculation.

and then dig under into the periphery of the diseased portion. If the tissues are rather dry the bacteria may be forced into the cavity by careful squeezing, or some drops (loops) of sterile water or beef-bouillon may be introduced into the cavity and stirred around before the bacteria are removed. If heat is inadmissible, the specimen may be washed or soaked for a time (15 seconds to 60 minutes) in mercuric chloride water (1:1000) and the surface thus freed from many contaminating organisms. Carbolic acid (5 per cent in water) or lysol (5 per cent in water) may also be used for sterilizing surfaces. Of course these substances must be removed as far as possible before the surface is broken. This may be done to some extent by swabbing with sterile absorbent cotton dipped into sterile water or by plunging into sterile water and shaking. The disinfectants will be more certain to touch and sterilize every part of the surface if all adhering particles of air are driven off by first plunging into alcohol for a moment.

In case of bacterial leaf-spots the writer generally obtains satisfactory cultures by cutting out the spot and plunging it for a few seconds (15 to 45) into 1:1000 mercuric chloride water, then rinsing in sterile water for a few minutes, crushing and throwing into a tube of bouillon from which the plates may be poured in course of an hour, *i. e.*, as soon as the bacteria from the interior of the spot have had time to diffuse into the bouillon. I frequently crush with a sterile glass rod, after throwing the material into a tube of bouillon, or else on a small sterile cover-glass which is then thrown into the bouillon.

In cases where heat and chemical disinfectants are both inadmissible on account of danger of destroying the organisms within delicate tissues, as in thin leaves and other soft parts, the bacteria or fungus-spores accidentally lodged on the surface may be greatly reduced in number by gently rubbing all parts of the surface between the thumb and finger under distilled water and then washing them in three or four successive beakers of distilled sterile water, the fragments being transferred from one beaker to the other by means of sterile forceps. Of course, the thumb and fingers must be well cleaned in advance by scrubbing and sometimes by the use of alcohol and corrosive sublimate, followed by sterile distilled water. When dry, these washed specimens may be scraped into, directly for plate cultures, or after the epidermis has been peeled off with cold sterile knives and forceps.

Quantitative determinations may be made by grinding up a given quantity of the suspected plant tissue, *e. g.*, a cubic centimeter or a gram, in a sterile mortar with clean sterile sand and 10 or 20 cc. of beef-broth or sterile water, and then making plates from carefully measured portions of the fluid, *e. g.*, from one 2-mm. loop, from 0.1 cc., 0.5 cc., etc. A like number of check plates made from equal portions of healthy tissues ground under precisely similar conditions will soon demonstrate about how many colonies are to be expected per plate (and what kind) as the result of surface contamination or air-borne bacteria introduced during the process of grinding.

The procedures described under *c* and *d* should be repeated a number of times (the more the better) and always with uninoculated plants in abundance for comparison. *These control-plants or check-plants must remain healthy.* If they also become



diseased, then the experiments must be done over with more care and times enough to remove all possible chance of error. When check-plants become diseased, especially in any number, there is always room for grave suspicion. Either the experimenter has been grossly careless, assuming that he used the right organism in his inoculation-experiment, or else he is working in a locality where the cause of the disease is naturally abundant. In either case, however well convinced he himself may be, his readers will generally have a lingering suspicion that even his inoculated plants succumbed not to what he inserted into them, but to some entirely different cause naturally present and overlooked by the investigator. The remedy for the

first is to learn to use infectious material with more caution, and for the second is to make the inoculation-experiments in localities or under conditions where the plant shall be less subject to natural infection.

If the experiments must be performed in localities where the disease is naturally present, then a large number of plants must be selected for inoculation and for control, and such a high percentage of infections secured in the inoculated plants that the few cases occurring naturally in the control-plants may be neglected as not casting any doubt on the general result. For example, if, in a region subject to the given disease, 100 plants were reserved for control and 100 similar plants were inoculated, and out of this number 50 of the latter and 40 of the former should contract the

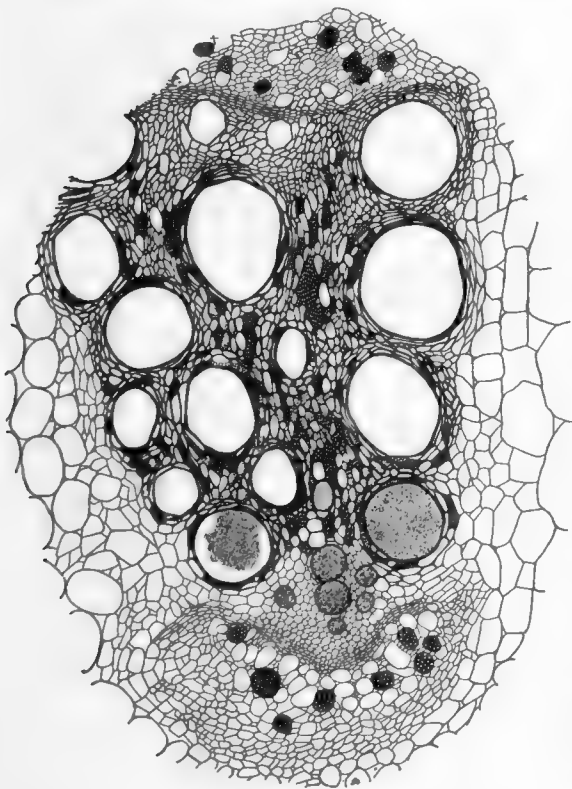


Fig. 9.\*

disease, it is manifest that no deductions of any value could be made from the experiment. All might be the result of some cause totally different from the

\*FIG. 9.—Cross-section of a small part of a cucumber stem, showing the parasitism of *Bacillus tracheiphilus* in one of the inner bundles. As yet there is no bacterial cavity, the bacilli being confined to the spiral vessels and a very few of the adjacent pitted vessels. Material taken from a field near Washington, D. C., in 1893. Sectioned from paraffin. Drawn from a photomicrograph.  $\times 50$ . Introduced for comparison with plate 3. Beginning at the top, the tissues occur in the following order: (1) Outer phloem, showing sieve plates; (2) cambium; (3) immature xylem; (4) mature xylem, consisting of pitted vessels and pitted connective tissues; (5) spiral vessels embedded in non-lignified living parenchyma, which is finally destroyed by the bacteria; (6) pseudo-cambial layer; (7) inner phloem; (8) large-celled parenchyma to either side, separating this bundle from its neighbors.

assumed cause, the different number of cases in the two groups of plants being accidental variations. If, in such a locality, only a very few plants are inoculated and a few held as checks, the evidence becomes still weaker and would not be considered entirely conclusive even though all of the inoculated plants should contract the disease and all of the checks should remain free, since in a region subject to a given disease five or six healthy plants may sometimes be found in proximity to five or six diseased ones, although all may have appeared healthy earlier in the season. The case is quite different if out of 100 control-plants and 100 inoculated plants 95 per cent of the latter and only 2, 5, or 10 per cent of the former contract the disease. It then becomes a question of probability which may be converted into reasonable certainty by several repetitions of the experiment with like results. Of course, the ideal experiment is one in which all the inoculated plants contract the disease and none of the control-plants, and in which a large number of plants has been used so as to exclude all possibility of the results being due to anything but the organism used.

Whenever the disease occurs naturally in the vicinity selected for the experiments, too much emphasis can not be laid on the necessity of having numerous inoculated plants and numerous controls, and on the desirability of repetitions of the experiment in different years and under different local conditions. It is important also that the inoculated plants should be under healthful conditions, *i. e.*, under conditions as nearly natural as possible. For example, proper (natural) conditions would be much more nearly attained by inoculating vigorous plants growing in the open air or in well-kept greenhouses than by inoculating parts of the same plants cut away from the stems and kept under bell-jars. It is conceivable that inoculations which would succeed very well under the conditions last named, especially at abnormally high temperatures, might entirely fail when under a more natural environment.

Not one of these four requirements can be omitted safely. A chain of evidence is not stronger than its weakest link. *Particular stress, therefore, is laid on being able to produce at will the characteristic signs and lesions of the disease in healthy plants by inoculation with pure cultures of a given sort; also on the re-isolation of the organism from the artificially-infected plants after they have become diseased; on the subsequent proper behavior of the organism in nutrient media; and on its ability to produce the disease when again inoculated.* This is the whole thing in a nutshell. The experiments must be continued until there is no doubt whatever as to the pathogenic or non-pathogenic properties of the organism. "Almost certainly pathogenic" always leaves room for grave doubt in the mind of every thoughtful reader. As a rule, the re-isolations should be made at a considerable distance from the point of inoculation, particularly if there is any doubt whatever as to the identity of the physical signs, since saprophytes have been known to live in plant tissues for a considerable number of weeks near the place of inoculation, and, if abundant, might cause various disturbances of nutrition without being the pathogenic organism sought for. For example, one would be more likely to obtain the cause of the disease in pure culture by attempting isolations from a plant in the stage shown in



*Datura metelloides* inoculated by needle-pricks with *Bacterium solanacearum* (Erw. Sm.).

The stems were pricked at O and O' on July 14, 1903, and the photograph was made July 22. The first signs of wilt appeared the 4th day. About one-third natural size.



plate 4 than from the same plant a week later (fig. 10). One would also be more certain of pure cultures by plating from the interior of the plant at A, B, or C, rather than at X or Y.

The judgment of experienced bacteriologists as to the pathogenic nature of



Fig. 10.\*

an organism may to a certain extent be accepted in absence of full proof, but only for the time being. Nothing is absolutely certain which has not been experimentally demonstrated.

\*FIG. 10.—*Datura metelloides*, inoculated by needle-pricks with *Bacterium solanacearum*. The same as plate 4, but six days later, i. e., on July 28.

If all experimenters in plant pathology, even in recent years, had been careful to conform to these four rules of practice, the first three of which in essence were formulated by Robert Koch as long ago as 1882, some very deep chagrins might have been avoided.

Owing to insurmountable difficulties many animal pathologists, especially those who study human diseases, now frequently rely on the first two rules as sufficient, but, if possible, one should comply also with *c* and *d*. Plant pathologists are under no such limitations, and should conform to each one of the above-mentioned requirements, particularly if they desire their work to take high rank and to be generally accepted as conclusive. Material for plant-inoculation experiments is so cheap and easily procured that a writer who undertakes to describe a bacterial disease of plants has usually no good excuse for leaving any doubt whatever as to the pathogenic properties of the organism. There is also no excuse for limiting the inoculations to mixtures of bacteria or to crude material taken directly from the diseased plant, since every tyro in bacteriology now knows how to separate one organism from another in nutrient agar or gelatin by means of poured plates or Petri-dish cultures.



Fig. 11.\*

#### MORPHOLOGY.

#### SIZE, SHAPE, ETC.

The smallest observed bacteria are only a small fraction of a micron in diameter. Migula states that the stained rods of *Ps. indigofera* (Voges) Mig. from colonies 36 hours old measured only 0.18 by 0.06 micron. *Bacillus denitrificans* (Amp. & Gar.) Mig. is also a very small rod—1.0 to 1.5 by 0.1 to 0.3 micron, according to Migula. *Micrococcus progrediens* Schröter is said to be only 0.15 micron in diameter. The organism of peri-pneumonia isolated by Nocard & Roux is more minute. It is probable also that still smaller organisms occur, *i. e.*, so small as to be invisible under the highest magnifications. In this way are interpreted the results obtained by animal pathologists in the foot-and-mouth disease and in some other diseases. Photographs with ultra violet light may in the end render some service here. The

\*FIG. 11.—*a*, Capsule of organism obtained from black spot of the plum. Bacteria grown in Uschinsky's solution and stained by Ribbert's method; *b*, ropy Uschinsky solution from which *a* was made.

largest bacteria are several thousand times as bulky as the smallest. Errera has described a spirillum the largest specimens of which measured 23 to 28 by 3 to 3.4 micra ('02, Errera, Bibliog., X), and Schaudinn has described a bacillus the largest forms of which are 24 to 80 by 3 to 6 micra ('02, Schaudinn, Bibliog., XI).

In shape the bacteria vary according to genera and species and sometimes within the limits of the species, from globose cells or very short straight rods, through curved forms or spirals, to filaments which are many times the diameter of the organism. To what extent does form vary under changed conditions? With the eye-piece micrometer make careful measurements of unstained organisms taken from the host-plant and from cultures of various ages and kinds. There is frequently considerable variability in the size of individuals of the same species. Is the breadth more constant than the length? Does the size or shape as observed in the plant differ from that observed on culture media? How does the living organism differ in size and general appearance from the dead, stained one?

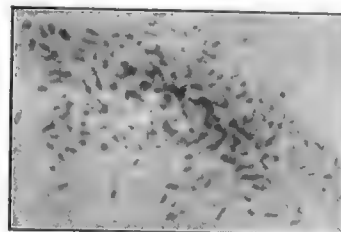


Fig. 12.\*

## CAPSULES.

The presence of capsules may be suspected whenever a bacterial growth becomes viscid. They are often difficult to see because their

index of refraction is so nearly that of the fluid in which they are usually examined. In examining unstained material the field should be illuminated with a narrow pencil of rays, and the effect of illumination with oblique light should be tried. Several methods of contrast staining are in use. By one method the capsule remains unstained or nearly so, while the central portion of the bacterium and the slime lying on the cover between the bacteria stain more or less deeply. By another method which has been spe-



Fig. 13.†

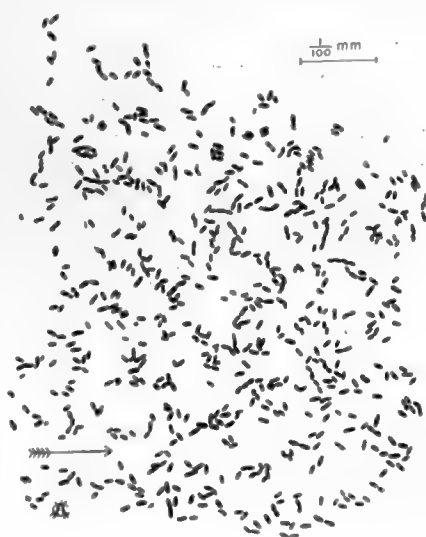


Fig. 14.‡

\*FIG. 12.—A portion of the yellow ooze from the black spot of the plum, stained by ordinary methods.  $\times 2,000$ .

†FIG. 13.—Cobwebby, sticky threads of *Bacillus tracheiphilus* drawn from the cut end of a muskmelon stem, arranged on a slide and stained with carbol-fuchsin. About three times natural size. Buzzards Bay, Mass., Oct. 8, 1903. Fig. 14 was drawn from the left-hand thread at the point marked X.

‡FIG. 14.—*Bacillus tracheiphilus* Erw. Sm. A portion of one of the threads shown in fig. 13. The arrow indicates the direction of the thread, which was extremely tenacious. The distance between the bacterial rods indicates very clearly the extreme viscosity of the unstained substance lying between them and holding them together.  $\times 1,000$ .

cially commended by Dr. Welch ('92, Bibliog., XIII), the capsule is also stained, but remains distinctly paler than the body of the bacterium. They may also be counter-stained, as in Muir's method or Moore's method. Well-defined capsules are shown in fig. 11a. This may be compared with fig. 12, in which the same organism is shown without capsules. Fig. 11b shows the extreme viscosity of a culture due to the formation and deliquescence of capsules. Fig. 13 shows the tenuous threads into which *Bacillus tracheiphilus* may be drawn as it oozes from the cut stems of cucurbits. Fig. 14 is a detail from the same more highly magnified, the viscid connecting substance being unstained.

#### FLAGELLA.

Ehrenberg was the first to describe flagella on bacteria (*Bacterium trilolare*, 1838). Nothing more was done until 1872, when Cohn discovered them on *Spirillum volutans*. In 1875 Dallinger & Drysdale saw and figured them on *Bacterium termo*. In 1875 Warming determined their existence on *Vibrio rugula* and *Spirillum undula*. In 1877 Koch demonstrated their existence on a number of species by the use of stains. In 1878 Dallinger, using unstained material, saw them many times on *Bacterium termo* and also on *Spirillum volutans*. After 1879 no one appears to have disputed their existence. In 1890 Messee proposed to divide the flagellate bacteria into four large groups, monotrichiate, lophotrichiate, amphitrichiate, and peritrichiate. In 1895 Fischer used the flagella as marks to distinguish subfamilies. In the previous year Migula used their number and mode of attachment as a means of distinguishing genera.

The staining of flagella has now become a regular part of laboratory work. Their number and position on the body wall should be determined, if possible, in case of each species studied. This is sometimes quite easy and at other times very difficult. It should also be determined whether the flagella are fugitive or persistent.

Flagella may be stained from young agar cultures. Bouillon cultures are to be avoided because of the intense ground stain. Some kinds may be stained readily from cultures grown for some days in a very dilute Uschinsky's solution—1 to 3 drops in 10 cc. of distilled water (fig. 15). The flagella of some bacteria are stained readily, those of others only with great difficulty. Many sorts seem inclined to throw off their flagella when transferred from agar to water. The cover-glasses must be *clean*. When cleaned ready for use seize with the forceps and pass them three times through the upper part of the Bunsen flame, with a considerable interval between each flaming, to avoid cracking. Use a minim quantity of the culture stirred in a big drop of water, or even in 2 to 10 cc. of water in a watch glass or test tube. Give the bacteria time to diffuse by waiting half an hour or more. Take the cover between the thumb and finger of the left hand, touch the end centimeter of a platinum needle to the water containing the bacteria, and sweep it deftly across the cover glass. In this way the fluid is spread in a very thin sheet over nearly the whole surface of the cover and is dry almost at once, with the bacteria well separated. If the fluid will not spread, then the cover is not clean and should be discarded. The bacterial sheet may be mordanted and stained at once, or first fixed by gentle heat. To avoid scorching, the cover should be held between thumb and finger when it is passed rapidly through the flame. Beginners usually burn the bacterial layer.



Smeary dark lines and other deceptive artefacts must not be mistaken for the flagella. The following methods have been tried by the writer and have given good results, but none can be depended upon always, and much time and patience are sometimes required to get good preparations of a refractory organism: Fischer's



Fig. 15.\*

modification of Loeffler's stain; Moore's modification of Loeffler's stain; Van Ermengem's nitrate of silver method; Löwit's copper-sulphate fuchsin mordant, followed by Ehr-

lich's anilin-water gentian violet. (For other methods consult "Formulæ" and "Bibliography of General Literature," XII.)

In connection with flagella-staining a white porcelain tray, such as photographers use, will be found very convenient for washing, and also the double blow-bulb shown in fig. 17. This should be attached to a wash-bottle, such as that shown in fig. 16. This will deliver a small stream, very good for washing excess of mordant and stain from the covers. To furnish a steady stream the bulb has to be compressed only about once a minute. The flask used for this purpose should hold a liter.

#### SPORES—ENDOSPORES, ARTHROSPORES.

Do arthrospores really occur? If so, in what respect do they differ from the ordinary vegetative rods? Test spores for resistance to high temperatures in the

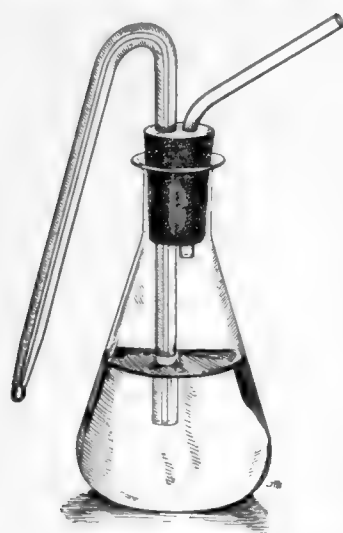


Fig. 16.†

water bath and to steam heat; study germination in hanging drops. Do the spores require a period of rest or refuse to germinate except in special media? The suspected existence of spores may be definitely settled by seeing the problematic bodies germinate. In the absence of such proof, considerable certainty may be reached by a combination of two methods: (1) the use of watery basic anilin stains, and (2) the use of moist heat. If at room temperatures the glistening bodies refuse to take the simple stains even on long exposure and at the same time are very resistant to steam heat or to hot water, *i. e.*, much more so than the ordinary vegetative rods, it may be assumed that they are spores. If, on the contrary, they are destroyed by temperatures only slightly above the recorded thermal death-point of the vegetative rods, it must not be assumed that they are spores, no matter how they behave toward

\*FIG. 15.—Flagella of yellow organism plated from black spot of plum. Stained from culture grown in 10 cc. distilled water containing a few drops of Uschinsky's solution.  $\times 1,000$ .

†FIG. 16.—Beyerinck's drop-bottle. The size and number of drops in a given time are regulated by sliding the bent tube through the cork. It is very convenient to have this flask on the microscope table. By a minim infection of the fluid it may also be arranged so that each drop shall deliver a single spore or bacterium for hanging-drop studies. About two-fifths natural size.

stains, unless they can be made to germinate. Many of the older identifications of spores are untrustworthy. Alfred Fischer has shown that many of these determinations rested on plasmolysis of the rods, *i. e.*, on misinterpretations. Omélianski reports finding an oval spore which stains readily with ordinary anilin stains. This occurs in a rather large bacillus accompanying his hydrogen cellulose ferment. Darnapple reports finding spores which are very sensitive to heat ('99, Bibliog., XXXIII). Usually only one endospore occurs in each cell, but Kern ('81, Bibliog., VIII), and Schaudinn ('02, Bibliog., XI) have found bacteria with two in each cell. Excellent directions for the study of spores are given in Part I of Migula's *System der Bakterien* (see especially the second paragraph on p. 209).

#### CELL-UNIONS—ZOOGLÆÆ, CHAINS, FILAMENTS.

In some media bacteria are much inclined to separate after division; in others they remain attached in various ways. The most common method of union is an irregular clumping, which in fluids gives rise to a fine or coarse flocculence. Such unions also occur on solid media and may be designated zooglææ, or *pseudo-zooglææ*, if one prefers to retain zooglææ for the more intimately fused and compacter gelatinous unions. Sometimes the organisms remain attached end to end. Where the segmentation is distinct, such unions are designated chains. When very long and with obscure segmentation, they may be called filaments. Is there any true branching? What especial conditions of the culture medium favor the formation of zooglææ, of chains, and of filaments? Many bacteria form zooglææ, chains, or very long filaments under certain conditions, while under other

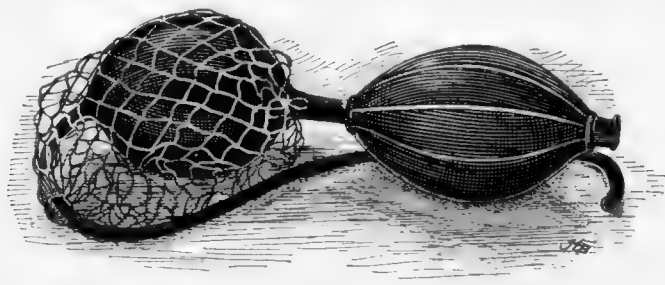


Fig. 17.\*

conditions they remain as very short, straight rods. (Compare figs. 18 and 19.) As in case of involution forms unfavorable cultural conditions (thermal, nutrient, etc.) appear to have much to do with their appearance.

The growth of bacteria may be studied in hanging drops of bouillon, etc. Hollow-ground slides (fig. 20) should be used for this purpose, rather than ring-cells, especially with high powers. Hill's hanging-block method is also serviceable ('02, Bibliog., XVII).

\*FIG. 17.—Double blow-bulb for attachment to drop-bottle shown in fig. 16. By use of this device one obtains with a minimum of pumping a constant small stream of water very suitable for washing stained covers, etc. Made by Emil Greiner. It is best used with a larger flask than that shown in fig. 16. Bulbs which have been long in stock should not be purchased, as the rubber deteriorates rapidly.

## INVOLUTION FORMS.

Under this name we designate swollen and distorted forms common in old cultures (fig. 21). Under what conditions do they occur? Are they living or dead? Isolate in hanging drops of bouillon and determine whether they are stages in development or only degeneration forms. Are Y-shaped or branched forms such as occur in old cultures of *B. tuberculosis* Koch, and in the root-tubercles of clover (fig. 22) to be considered as involution forms? Are such organisms fungi or bacteria? Branching forms have been detected by many observers. (Consult numerous citations in the Bibliography of General Literature, X). The most recent paper is by Albert Maassen (Arb. a. d. Kais. Gesundh., Bd. XXI, H. 3,

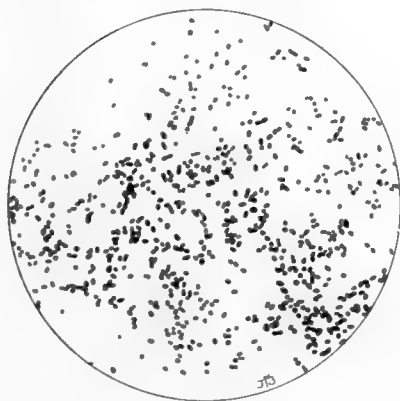


Fig. 18.\*

1904, p. 377, 6 pl.). He found chloride of lithium specially advantageous for provoking these growths, which are regarded as teratological. He obtained them in 24 hours.

## GENERAL COMMENT.

Great care should be paid to the minute morphology of each organism, not only in the host-plant but also in a variety of cultures, old and young, so that a body of knowledge more exact than we now possess shall be gradually accumulated for differential and systematic purposes. Careful drawings and photographs should be made. The Abbe camera is a great help in making drawings (fig. 121). For such study the Zeiss apochromatic lenses and compensating oculars can not be recommended too highly, particularly the 16 mm., with the 12 and 18 compensating oculars for studying the margins of colonies, and the 2 mm. 1.30 n. ap., with the 8 and 12 compensating oculars for the more detailed study of the individual rods. The writer has also made much use of the Zeiss 3 mm. 1.40 n. ap. apochromatic objective. The Zeiss screw, or filar, micrometer combined with a No. 12 compensating ocular (fig. 23) will be found very useful. For photographic purposes the projection oculars or the 4 or 6 compensating oculars may be used. Robert Koch was entirely correct in saying: "A general use of photography in microscopic works would certainly have prevented a great number of unripe publications."



Fig. 19.†

\*FIG. 18.—*Bacterium campestre*. Cover-glass (smear) preparation from the vessels of a cabbage plant received from Racine, Wis., Sept. 19, 1896. Stained with carbol-fuchsin. Drawn from a photomicrograph.  $\times 1,000$  circa.

†FIG. 19.—*Bacterium campestre* from an old culture on 23 per cent grape-sugar agar, showing long filaments. Cover stained 1 hour and 20 minutes in gentian violet (1 part saturated alcoholic solution plus 1 part water). Many of the rods stained feebly. Tube inoculated June 30, 1898. Cover prepared Aug. 8. Drawn directly from the slide.  $\times 1,000$ .

Good photomicrographs should be secured if possible. Koch's first photomicrographs were of various enlargements. He afterwards recommended  $\times 1,000$  as the standard magnification, but  $\times 1,500$  and  $\times 2,000$  are also convenient sizes and occasionally  $\times 500$  is better than  $\times 1,000$ . Most important is it that the exact magnification should always be indicated. The Zeiss apochromatic objectives are much better for photographic work than the achromatic ones. For very small magnifications the writer has found the old Zeiss 35 mm. and 70 mm. very useful. For the same purpose the newer Zeiss planars, series Ia Nos. 1-5 (fig. 122) are admirable. These have sharp definition and a very flat field, but not much depth of focus. With them objects several centimeters in diameter may be satisfactorily photographed with magnifications from 2 or 3 diameters to 50 or more. The writer obtains as sharp a focus as possible with wide-open diaphragm and then stops down about two-thirds.



Fig. 20.\*

One of the best simple photomicrographic outfits is the Zeiss upright camera (fig. 24). All apparatus is to be rejected which requires the microscope to rest on the same platform as the camera. It should rest on the table independent of

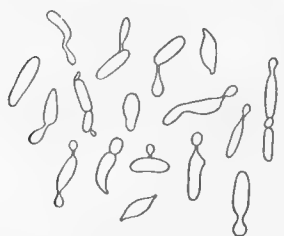


Fig. 21.†

the camera, unless a weak light is used and the exposures are very long, in which case a slight jarring is of no great consequence. Direct sunlight is the best light, but the light of the open sky may be used (with full open diaphragm) if one is willing to make 5 to 20 minute exposures. Electric light is often used by those who live in cloudy regions or who occupy rooms not exposed to the sun, but the writer has had no experience with it.

Very good pictures also may be made by gaslight if the

Welsbach burner is used. Ordinary lamp light (kerosene) is too yellow and not sufficiently intense. Photographs can be made with a kerosene light, but the time and trouble involved make it scarcely worth while to consider this source of light. The writer has obtained the best results by using direct sunlight and slow isochromatic plates behind Zettinow's light filter. Of course, with upright cameras a dry light-filter must be used, such as the yellow one devised by Carbutt or by Ives. In using a horizontal apparatus, such as that shown in plate 5, the *sine qua non* is to get it properly leveled up and to keep it so.

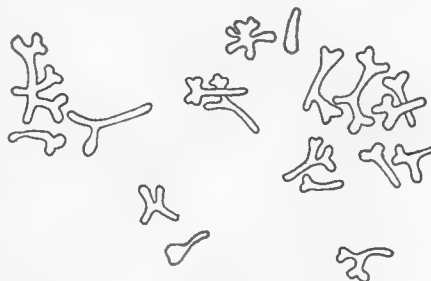


Fig. 22.‡

\*FIG. 20.—Hollow-ground slide with cover-glass bearing hanging drop for examination under the microscope.

†FIG. 21.—Involution forms of *Bacillus tracheiphilus* from extremely ropy potato broth. Drawn free hand,  $\times 1,000$  circa. Many as large as 8 by 2 micra and others larger. Nov., 1894.

‡FIG. 22.—Y-shaped (dichotomously branched) bodies from the root-tubercles of clover (*Trifolium*). From a photomicrograph by the author, made from a slide furnished by Dr. Geo. T. Moore.  $\times 1,500$ .

For the inspection of colonies and of subcultures in tubes the best hand-lens known to the writer is the Zeiss aplanat magnifying six times (fig. 25). That magnifying 10 times is also very useful, but will not reach to the center of an ordinary test tube. Those in apple-tree wood cases are in some respects more convenient than those provided with metallic swing covers (fig. 26).

The best general work to consult on the morphology of the bacteria is undoubtedly Migula's System (see Bibliog., III).

#### PHYSIOLOGY.

In the description of bacteria we are compelled to make large use of physiological peculiarities, owing to their very simple and monotonous morphology. Within the limits of the genera now recognized the form differences are so very slight



Fig. 23.\*

that many bacteria, *e. g.*, *Bacillus coli*, *B. cloacæ*, *B. suipestifer*, *B. typhosus*, *B. amylovorus*, etc., are indistinguishable under the microscope. In mixed cultures, or stained preparations, no one could distinguish one from the other with any certainty, and in pure cultures of unknown origin certain identification by means of the microscope would be equally impossible. Nevertheless, these same forms are so widely different in their behavior in culture media, in their pathogenic properties, in their relation to heat, air, antiseptics, etc., that we are certainly warranted in regarding them as distinct species, using the word "species" in its common acceptation. These well-ascertained facts should not, however, lead one to neglect slight differences of form, even when they can be expressed only in fractions of a micron. On

\*Fig. 23.—Zeiss compensating ocular No. 12 with screw-filar micrometer.

the contrary, as much as possible should be made out of morphology, particularly that of the living organism, and in this connection the recent efforts of Migula and Fischer are especially deserving of commendation.



Fig. 24.\*

\*Fig. 24.—Upright Zeiss camera for photomicrographic work. The cup (a) slips over the end of the microscope and forms a light-tight connection with the bellows without touching it. The microscope rests on the table independent of the camera. The stout rod turns freely in the socket X and is locked in place by a set-screw on the side opposite the observer. The height is about 45 inches.

†Lehmann and Fried (Arch. f. Hyg., Bd. XLVI, 1903, p. 311) found the swiftest movement of bacteria to be 1 mm. in 22 seconds; the slowest 1 mm. in 222 seconds; average: cholera, 1 mm. in 34¼ seconds; typhoid, 1 mm. in 56 seconds; *B. vulgare*, 1 mm. in 73 seconds; *B. subtilis*, 1 mm. in 40 seconds; *B. megaterium*, 1 mm. in 2 minutes 11 seconds.

#### MOTILITY.

If motile, determine kind of motion and rapidity (margin of small hanging drops on thin covers suspended over hollow-ground slides).† The cover may be prevented from sliding by touching one edge with a *very little* vaseline or cedar oil; if too much is used it runs under, mixes with the hanging drop, and spoils the mount, and possibly in the end the objective is ruined, if the student continues to search for a clear field. The beginner is very apt to mistake Brownian movement for self-motility. It sometimes requires very careful observation to be quite certain. Rods which appear to be motionless will sometimes be seen to dart away quite suddenly if watched. In some species young cultures are much more apt to be motile than old ones; in others motility appears to be an almost constant characteristic. The movements of bacteria are sometimes quite characteristic for particular sorts. They may be slow or rapid tumbling motions centering in the shorter axis, or straight or sinuous slow or rapid darting movements in the direction of the longer axis, with rotation on this axis. The media of Hiss ('97, Bibliog., XVI) and of Stoddart ('97, Bibliog., XVI) are sometimes useful for distinguishing macroscopically between motile and non-motile forms. The former spread as a thin layer over the whole surface, the latter pile up in restricted areas around the points of inoculation. The student should not remain content with merely determining motility, but when this has been settled he should turn his attention to staining the organs of motion.



Large horizontal Zeiss photomicrographic outfit ready for use, except that when photographing the curtain is raised and the mirror is placed farther away, i. e., out of the south window on the triangular extension shown on the front table at the right. In the newer forms each table top may be raised or lowered at will. There is also a device for raising or lowering the plate on which the microscope rests.





## GROWTH.

The manner of growth and rapidity of growth at given temperatures in hanging drops and also on the margin of young colonies on plates of nutrient gelatin and agar of varying density should be determined. Frequently characteristic and interesting arrangements of the rods forming the surface layers of the colony, especially when it is young, may be discovered by means of a direct inspection of the colonies under low powers of the microscope or by means of cover-glass impressions. Covers are carefully placed on the colony, removed, dried, flamed, and stained. There are also often curious arrangements of the deeper layers of the



Fig. 25.\*

surface colony. In direct examination the colonies should be viewed by reflected as well as by transmitted light. Drawings or photographs of surface colonies should be made under low or medium magnifications. By a little practice using Lister's dilution method ('78, Bibliog., XVII), hanging-drops containing a single bacterium for study under the microscope may be obtained with Beyerinck's capillary drop-flask ('91, Bibliog., XVII).

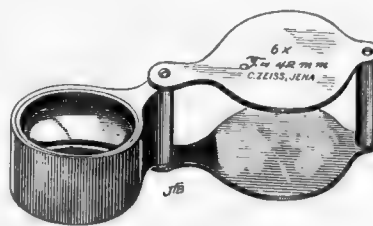


Fig. 26.†

## CHEMOTROPISM.

On the general subject of chemotropism, see papers by Pfeffer, Miyoshi, Jennings, Buller, Rothert, etc. Jennings maintains that contact irritation inducing motor reflex is responsible for movements which were formerly attributed to chemical stimulus. Consult Jennings, "Contributions to the study of the behavior of lower organisms," Carnegie Institution of Washington, 1904, and especially Jennings and Crosby, "The manner in which bacteria react to stimuli, especially to chemical stimuli," Am. Jour. Physiol., Vol. VI., pp. 31-37, and Jour. Roy. Mic. Soc., 1902, p. 88. *Spirillum volutans* was used in the tests.

## REACTION TO STAINS.

Proper staining is a very important part of the study of bacteria. Its foundation principle is the fact that the bacteria, in a living vegetative condition, all show a great affinity for the basic anilin dyes. Spores ordinarily show no such affinity, but may be made to take up stains by acting on them with strong acids or alkalis, or by heating them very hot. Flagella also show no affinity for stains until acted

\*FIG. 25.—Hand lens suitable for examining bacterial cultures. Zeiss aplanat magnifying six times. Three-fourths natural size.

†Fig. 26.—Zeiss swing-cover aplanat magnifying six times. This is now sent out in a neat little chamois-skin purse. About two-thirds natural size.

on by severe reagents, when they may be stained in mordanted solutions or in dyes which have been preceded by a mordant. The outer wall of the bacterium generally reacts to stains in the same way as the flagella, *i. e.*, it usually remains unstained.

Staining media may be roughly divided into four groups: (*a*) Simple stains dissolved in water, *e. g.*, fuchsin (basic), gentian violet, methylene blue; (*b*) alcoholic solutions and various complex stains, *e. g.*, saturated alcoholic solutions of anilin dyes, alcohol-iodine, iodine potassium iodide, Russow's cellulose test, Ziehl's carbol-fuchsin, Loeffler's alkaline methylene blue, Ehrlich's anilin-water gentian violet, Gabbett's stain, Gram's method, Delafield's hæmatoxylin, Ehrlich's acid hæmatoxylin, Heidenhain's iron-hæmatoxylin, Fleming's triple stain; (*c*) flagella and capsule stains, *e. g.*, Loeffler's stain, Moore's modification, Fischer's modification, Bunge's stain, Löwit's stain, van Ermengem's nitrate of silver method, Zettnow's gold method, etc.; (*d*) stains for spores, *e. g.*, prolonged exposure to simple stains

dissolved in water (which should have little effect), steaming carbol-fuchsin with methylene blue for contrast, etc. (see "Formulæ" and Bibliography of General Literature under "Flagella," "Spores," etc., for various observations on staining).

Grübler's stains are preferred. Cover-glasses should be clean and free from fat, traces of which are easily removed in a Bunsen flame. A little experience is necessary in flaming thin covers in order not to crack them. They may be passed through the flame three times, waiting a moment or two after each pass for them to cool. The student should see that the water used in making the cover-glass preparations or the



Fig. 27.\*

stains does not itself contain bacteria. It is usually wise first to dry a drop of the water on the cover and stain without addition of the bacteria. Eternal vigilance is the price of trustworthy results. It is best to make all mounts on cover-glasses of a known and uniform thickness (0.15 mm.). Many a good preparation has been spoiled for examination with lenses of a short-working distance by mounting under a thick cover-glass, and sometimes the lens itself has been ruined in the attempt to focus. The thickness of covers often varies greatly from the statements of dealers, and they should not be accepted until tested with a reliable cover-glass measurer (fig. 27).

\*FIG. 27.—Zeiss cover-glass measurer. The cover in place shows a registered thickness of 0.18 mm. Fractions of an inch are also registered on this instrument.

To determine whether bacteria are properly stained examine with the diaphragm of the condenser wide open. If they can not be seen distinctly with this flood of light they are not well stained. The bacteria should be well separated on the cover and deeply stained, while the background should be very free from stain.

Dr. Weigert seems to have been the first to use anilin stains for the demonstration of bacteria in tissues. This was about 1875. Since that time staining in tissues has been worked up carefully for bacteria causing animal diseases, but very little is known respecting best methods of staining bacteria in vegetable tissues. The difficulty lies in the fact that the tissues of the higher plants often take the basic anilin stains as readily as the bacteria and retain them even more tenaciously. Special remarks may be looked for under particular diseases.

#### CULTURE MEDIA.

##### NUTRIENT GELATIN.

(a) *Plate Cultures*.—Colonies, young and old, buried and superficial, crowded and wide apart, should be examined for color, translucency or opaqueness, shape, thickness of the surface growth, and character of the margin. They should also be studied under low powers of the compound microscope for lobes, branches, granulations, wrinkles, flecks, concentric rings, radial filaments, arrangement of the dividing rods on the margin of the colony, iridescence, etc. The microscopic appearance of the surface colony during the first 48 hours is often different from that later on. The rapidity of growth should be compared with that of some common and easily accessible organism, *e. g.*, *Bacillus coli*, *B. amylovorus*, *Bacterium campestre*. The comparative rate of growth of buried and surface colonies should also be carefully noted. How is the appearance of the colony changed by increasing the amount of gelatin, or varying the brand of gelatin? Are the surface colonies viscid, or can they be lifted bodily in one mass from the substratum?

(b) *Stabs*.—The nature of the surface growths and of the deeper growths should be carefully examined. Is there any marked tendency of the latter to grow downward or outward into the body of the gelatin, either in distinct masses or as a diffused cloudiness? Observe effect, if any, on growth when the gelatin is acid or only feebly (litmus) alkaline. If liquefaction of the gelatin occurs, note its rapidity and whether it is mostly restricted to the surface or is equally rapid along the line of the stab in the depths; note also whether the liquefied gelatin is clear or cloudy in tubes which have not been shaken, and whether a pellicle has formed on its surface. Liquefaction may be very rapid (taking place within a few hours), may occur after three or four days, may be long-delayed and feeble (only visible after some weeks), or may not occur at all. It is the cases of feeble and long-delayed liquefaction which lead to contradictory statements on the part of different observers, and consequently cultures should remain under observation for a considerable time and on a variety of gelatins. Various substances interfere with liquefaction. Determine whether liquefaction can be prevented by the addition of grape-sugar or cane-sugar (10 per cent). Look for gas-bubbles, for crystals, for any fluorescence or staining of the medium (green, brown). Inasmuch as the growth of some bacterial plant

parasites is restrained by some nutrient gelatins which are neutral or only feebly alkaline to litmus, it is advisable to add to a part of the stock more caustic soda than is commonly used, *i. e.*, enough to render it neutral to phenolphthalein (strongly alkaline to neutral litmus), especially if gelatin is selected as the first medium for isolation experiments; otherwise perplexing failures may result.

(c) *Streaks*.—Record the character of the streak, whether wet or dry, smooth, wrinkled, or rough, thin or piled up, margin well defined or indistinct. Note also whether the surface is ever iridescent, whether growths are sent down from the under surface into the substratum, whether the streak spreads rapidly and widely over the

surface or very slowly. The surface behavior depends to some extent on the motility of the organism, on the amount of water in the surface layers, *i. e.*, whether the slants are fresh or old, and on the amount of gelatin in the medium, which in temperate climates should usually be 10 per cent, but may be 15 or even 20 per cent. By minimizing heat in preparation and by increasing the quantity of gelatin to 20 or 30 per cent a medium may be obtained which will remain solid at 30° C. Growth is less satisfactory, however, on such a dense medium, or at least was in the few tests made by the writer. Chester has applied the ordinary botanical terminology to the varying margins of colonies, etc., and has published some useful figures ('or, Bibliog., III).

No substance used in the bacteriological laboratory is so uncertain



Fig. 28.\*

tain and variable in its composition as gelatin. The gelatin from different factories varies greatly and hardly any two batches from the same factory are alike. One glue chemist has defined gelatin as "80 per cent glue, 10 per cent dirt, and 10 per cent doubt." It varies greatly in its melting point and power of setting, and in amount of peptones and albumoses it may contain, which is sometimes large. It always contains calcium salts and phosphates, which are often antiseptic, and the nature of which varies according as hydrochloric or sulphurous acid has been used in its manufacture. Formaldehyde is sometimes added to it, we are told; and occasionally agar also, it is

\*FIG. 28.—Nelson's photographic gelatin No. 1. Recommended for bacteriological use.

said, is added to certain table gelatins to increase their body. Gelatin also contains a variety of decomposition products due to the growth in it of various fungi and bacteria while it is in the vats or in the drying-house. If there is any delay in the drying it is spotted all over with molds and bacteria. It also contains some wax or grease, used to anoint the surface on which it is spread to dry, and this wax or grease is probably also a variable substance. Gelatins also polarize, it is said, in many different ways. An absolutely pure gelatin of uniform character for bacteriological purposes is not to be had. That which perhaps comes the nearest to it and which is here recommended is Nelson's gelatin, made in London and well known to the makers of photographic dry-plates, who use it in large quantities. It comes in two grades, a hard and a soft, and costs about \$1.25 per pound. No. 1, that which I like best, comes in shreds resembling "excelsior" used for packing (fig. 28). No. 3, which comes in long, broad strips, contains much cell detritus, etc., and filters with difficulty. Other expensive gelatins, said to be of quite uniform quality, are

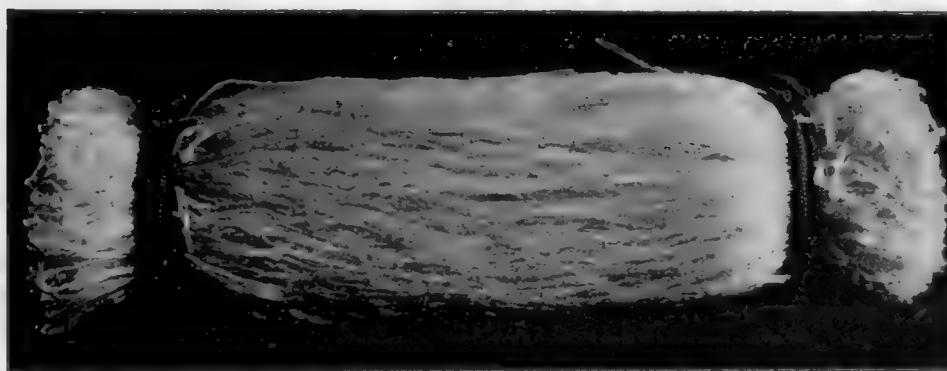


Fig. 29.\*

Lichtdruck gelatin, made by Carl Creutz, Michelstadt, in Hesse, and Geneva Red Cross gelatin made by Winterthur, in Switzerland, under direction of Dr. Eder, of the Imperial Institute of Vienna (Cockayne).

#### NUTRIENT AGAR.

Agar, or agar-agar, as it is usually called, from a Malay word meaning "vegetable," is a manufactured product obtained from various sea-weeds growing in Chinese and Japanese waters. Various species are used as food and the trade is considerable. It usually comes into the hands of the bacteriologist as long, slender, yellowish-white strips (fig. 29) or as blocks (fig. 30), or more especially in recent years, in the form of a gray-white fine powder of European manufacture (fig. 33). It is reputed to be the product of species of *Gelidium* (figs. 31 and 32).

\*FIG. 29.—The kind of agar-agar usually employed in bacteriological work. This is a manufactured product known to the Japanese as slender "Kanten." The figure represents first quality "Kanten," in unbroken package. (Courtesy of Dr. Hugh M. Smith, Deputy Commissioner of the United States Bureau of Fisheries, who brought the package with him from Japan.)

Of the Japanese algæ in this group the following, according to Rein (pp. 81-82), deserve special mention:

- |   |   |
|---|---|
| (1.) <i>Chondrus punctatus</i> Sur.                                 | (10.) <i>G. cartilagineum</i> Gail.   |
| (2.) <i>Gigartina tenella</i> Harvey; Jap. Ogo.                     | (11.) <i>G. rigidum</i> Grev.; Jap. Tosaka-nori, <i>i. e.</i> , cockscomb algæ. |
| (3.) <i>G. intermedia</i> Sur.                                      | (12.) <i>Sphaerococcus confervoides</i> A.; Jap. Shiramo.                       |
| (4.) <i>Gloiopeltis tenax</i> Kg. ( <i>Sphaerococcus tenax</i> Ag.) | (13.) <i>Gymnogongrus flabelliformis</i> Harv.; Jap. Home-nori.                 |
| (5.) <i>Gl. capillaris</i> Sur.; Jap. Shiraga-nori.                 | (14.) <i>G. japonicus</i> , Sur.; Jap. Tsuno-mata.                              |
| (6.) <i>Gl. coliformis</i> Harv.; Jap. Kek'Kai.                     | (15.) <i>Kallymenia dentata</i> ; Jap. Tosaka-nori.                             |
| (7.) <i>Gl. intricata</i> Sur.; Jap. Fu-nori.                       | (16.) <i>Porphyra vulgaris</i> Ag.; Jap. Asakusa-nori.                          |
| (8.) <i>Gelidium corneum</i> Lamouroux; Jap. Tokoroten-gusa.        |   |
| (9.) <i>G. Amansii</i> Lamour.                                      |   |

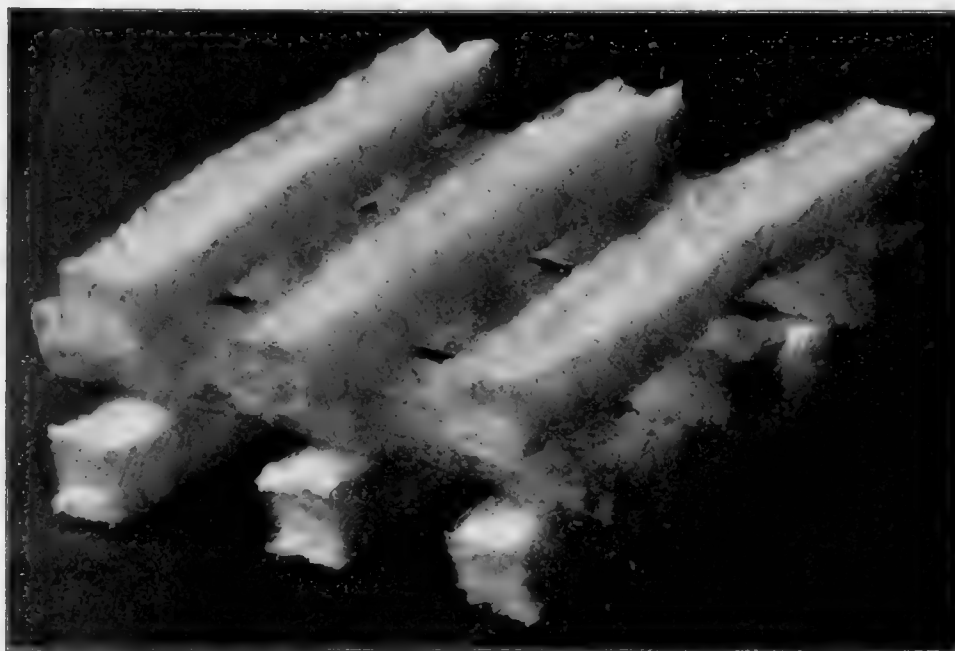


Fig. 30.\*

Agar-agar is a neutral or nearly neutral substance which is converted by boiling with water into a stiff jelly that hardens in 1 per cent solution at 39° to 40° C., and is not easily liquefied either by the growth of organisms† or by heat less than that of boiling water. It is a kind of vegetable glue forming a good matrix for various nutrient substances. A chemical analysis by Karten (Descript. Cat. Int. Health, Exhib., London, 1884) gave the following proximate composition: 11.71 per cent nitrogenous matter (albumen [?]), 62.05 per cent non-nitrogenous matter (evidently glue, the pararabin of Reichardt), 3.44 per cent ashes, and 22.80 per cent water.

\*FIG. 30.—Another form of agar-agar known to the Japanese as square "Kanten." The bulk of this goes to Holland, where it is used for clarifying schnapps. Courtesy of Dr. Hugh M. Smith. The actual size of these sticks is about 10¼ by 2½ by 1¼ inches.

†Metcalf has described a bacillus which slowly softens it, and the writer has observed similar phenomena.

For a full account of Japanese methods of making agar-agar consult a paper entitled "The Seaweed Industries of Japan," by Dr. Hugh M. Smith, in the Bulletin of the United States Bureau of Fisheries for 1904.



a



b

Fig. 31.\*

In addition to beef bouillon, or in place of it, various substances, organic and inorganic, may be added to the agar with advantage. The writer makes much use of *litmus-lactose agar*, which is made out of ordinary nutrient agar by adding 1 per cent milk-sugar and enough pure litmus water to give a purple-red color. *Glycerin-agar*, *maltose-agar*, etc., may be made up with any amount of the substance desired, generally 1 or 2 per cent.

Formerly it was difficult to filter agar perfectly clear and it was therefore used less than gelatin, but in recent years it has been discovered that this difficulty may be overcome if the agar is first brought into complete solution by prolonged boiling or by a short boiling at a temperature somewhat above 100° C., *e. g.*, 110° C.

The writer formerly obtained filtered clear agar by soaking the snipped agar in 5 per cent acetic-acid water for some hours, after which a thin cloth was tied over the mouth of the beaker securely, and tap water allowed to run into it for an hour or more *i. e.*, until all trace of acid was removed. The softened agar was then put into the bouillon, boiled for two hours, and finally filtered through S. & S. filter

\*FIG. 31.—Red sea-weeds from which agar-agar is manufactured. *a*, *Gelidium corneum* Lam., one-third natural size; *b*, *Gelidium subcostatum* Lam., one-half natural size. From a colored Japanese chart showing "The principal aquatic plants of Japan," supposed to be an official publication. Original in the library of the United States Fish Commission.

paper,\* using a hot-water funnel. Later he followed Schutz's method ('92, Bibliog., XVI), which is a very good one. This consists in cutting the agar into small bits and first heating it very hot in a beaker or enameled-iron dish in a minimum quantity of water or beef-bouillon over a hot Bunsen flame with constant and rapid stirring and



Fig. 32.†

\*The folded filter papers are the most convenient (fig. 34). These filter papers give the starch reaction (blue) with iodine, and reduce Fehling's solution on being boiled in it.

†Fig. 32.—Unnamed species of red sea-weeds (*Gelidium*) furnishing agar-agar. From a Japanese chart showing "The principal aquatic plants of Japan," supposed to be an official publication. One-half natural size. Original in library of United States Fish Commission.



occasional additions of small quantities of water until it is thoroughly cooked in the form of a thick mush. It is then put into the remainder of the water or bouillon and subjected to streaming steam for two hours, after which, if the first heating was sufficient, it filters readily without the use of a hot-water filter, or the necessity of keeping it in the steamer during the filtering. The stirring rod must touch all parts of the bottom of the dish exposed to the flame, every few seconds during the preliminary heating, otherwise the agar will burn on and be spoiled. On some

accounts it is best to begin operations with beakers rather than the enameled iron dishes. In this way all likelihood of using burned agar is avoided, since the moment the agar burns on the beaker cracks and the agar is spilled. For bacteriological use agar should be clear, not cloudy or filled with unremoved precipitates.

The writer now employs an autoclave and uses an agar flour procured from Lautenschläger or Merck (fig. 33). If one has an autoclave the preliminary heating of the agar in an open dish with a minimum quantity of water and all the subsequent stages may be dispensed with and the entire process carried on in the autoclave, unless it is known or suspected that media heated in the autoclave are less well adapted to the growth of particular organisms than those prepared at 100° C. The amount of agar added to the culture fluid is usually 1 per cent. On the making of nutrient agar

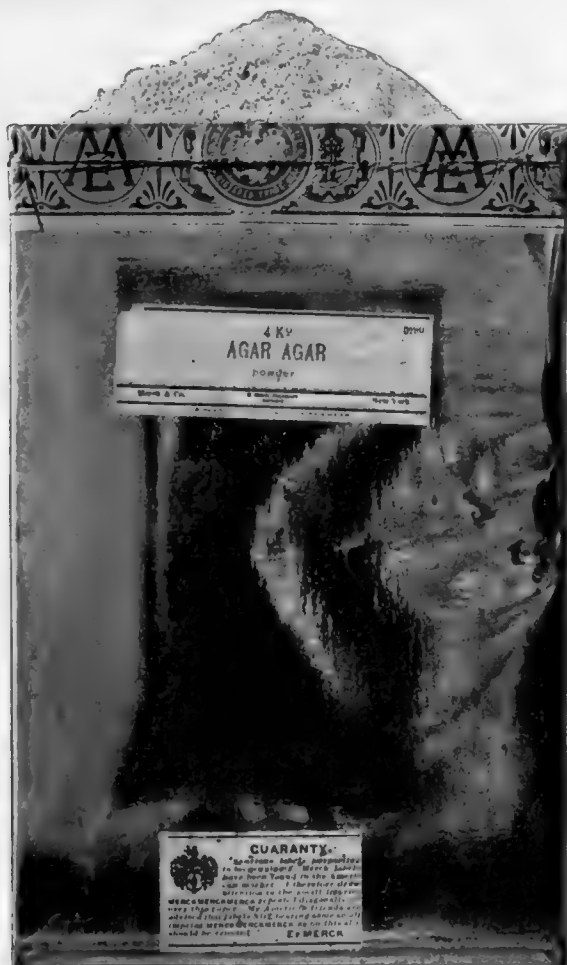


Fig. 33.\*

consult "Formulae," and the various standard text-books.

Is there any difference in the appearance of colonies when grown at 5° to 10°, 15° to 20°, and 30° to 37° C.? Observe the amount of precipitate that collects in the fluid in the V. For other observations as to growth on this substratum see "Gelatin." Every organism should be studied in numerous Petri-dish poured-plate

\*FIG. 33.—Agar-agar flour as received from European manufacturers. Package of Merck's agar flour.

cultures. Too many plate cultures can scarcely be made. Dishes with flat and very thin bottoms (0.3 mm.) are desirable for some purposes, but are difficult to procure. For quantitative work, plates with flat bottoms are necessary, and when photographs are likely to be wanted plates must be selected which do not have rings, wavy places or other flaws in the glass on the bottom. There is room for much improvement in the quality of the Petri dishes now on the market.

The student is advised to use agar media for all general laboratory work. When he has learned the behavior of an organism on nutrient agar, he may then try gelatin. Do any of the organisms under observation soften or liquefy the medium?

Agar roll cultures may be made in test tubes readily if the amount of fluid agar is reduced to one-half cubic centimeter.

When colonies are to be counted, special pains must be taken to distribute the gelatin or agar uniformly over the bottom of the dish.

Various persons—Pake, Jeffer, Weiss, Macé, et al.—have devised ruled plates for counting the number of colonies of bacteria in Petri-dish poured plates. The writer prefers to count by square centimeters or fractions thereof. When the plate is sown thin enough, the entire number of colonies should be counted. When it is very dense, the average may be taken of ten square centimeters selected with care, provided the bottom is flat, otherwise the whole plate must be counted. If the counting plate is to be placed under the dish, it may be opaque, *i. e.*, a black surface with white lines, not the reverse. If it is



Fig. 34.\*

to be placed on top of the dish, the latter preferably bottom up, then it should be of glass or some other transparent substance. The spaces may then be ruled on with a diamond, or drawn on in very fine black lines with India ink. The gelatin film of an unexposed, fixed photographic dry-plate is a very good surface for holding the ink. For counting colonies on very densely sown plates, the writer has found convenient a rectangle 20 mm. by 5 mm. divided into tenths.

#### SILICATE JELLY.

In recent years, in the hands of Winogradsky and his students, silicate jelly has played an important part in the isolation of various organisms, which do not take

\*FIG. 34.—Folded filter papers made by Schleicher & Schüll.

kindly to culture media containing animal and vegetable products. It is desirable also for exact experiment with other organisms. It may be used in Petri dishes or flasks, or slanted in test tubes. Along with some disadvantages, *e. g.*, tendency to split, it has a number of valuable characteristics, not least among which is the fact that it enables one to offer the organism a solid substratum which is at the same time purely synthetic. It is generally considered to be very difficult to make, but by following the most recent directions of Omélianski ('99, Bibliog., XXV), and especially certain slight modifications introduced by Moore & Kellerman and by the writer and his assistants, it can be prepared without difficulty, and to it may be added any mineral nutrient substances desired. The writer makes it in the following way:

To each 100 cc. HCl (sp. gr. 1.10° Beaumé) is added drop by drop 100 cc. sodium silicate (sp. gr. 1.09), the mixture being stirred continually with a glass rod. This is now placed in a collodion sack and dialyzed for some hours in running water. To this is then added in concentrated sterile form whatever synthetic culture medium is desired, after which the jelly is put into Petri dishes or test tubes and sterilized by heating for three hours in the blood-serum oven (fig. 45) on five consecutive days at 90° C., or by one steaming in the autoclave for 15 minutes at 110° C. The thermo-regulator shown in fig. 35 is useful for maintaining a constant high temperature in the oven. The oven must also contain some water in a capsule or beaker.

It is believed that a more detailed account of the manipulations connected with the preparation of silicate jelly will be welcome to many. First of all, one must have dialyzing sacks. Collodion sacks are much more convenient than parchment sacks, since they can be prepared at any time, and dialysis takes place through them with great rapidity. They are useful for so many purposes that material for making them should be on hand in every laboratory.

The writer follows Kellerman in making his sacks *inside* of test tubes. These may be large or small according to what the sacks are to be used for. If for dialyzing silicate jelly in some quantity, it is very convenient to make the sacks inside of test tubes 7 inches long and having an internal diameter of 1 inch. The first thing is to prepare the collodion mixture. This is made by dissolving soluble guncotton, such as is used by photographers, in a mixture of absolute alcohol and sulphuric ether. The writer uses equal parts of these two fluids. If too much alcohol is used, the sacks dry slowly, and if too much ether they are said to become brittle. After some



Fig. 35.\*

\*FIG. 35.—Tollen's thermo-regulator for maintaining blood-serum oven at 80° to 90° C. The stem and bottom of the bulb contain mercury. The remainder of the bulb is filled with glycerin. In the similar thermo-regulator used for the paraffin-bath chloroform replaces the glycerin. Actual height, 12 inches. Chloroform and glycerin are very useful in such thermo-regulators because their coefficient of expansion is much greater than that of mercury. Toluene may also be used with mercury.

experimenting it was found that 5 grams of the clean, white guncotton per 100 cc. of the fluid gave a solution very satisfactory to work with. About 24 hours is required to dissolve the guncotton into a homogeneous mixture, of which there should be at least 800 cc. This should be stored in a cork-stoppered bottle of shape convenient to hold in one hand. It is then ready for use. The clean test tube, thoroughly dry on the inside, is now held in one hand in a slanting position, mouth up, while with the other the collodion is poured slowly and steadily into the tube, while the latter is slowly rotated. In this way air-bubbles are avoided and the entire interior of the tube is moistened. When this has taken place and about an inch of fluid has accumulated in the bottom of the tube, the excess is poured back into the bottle, slowly rotating the slanted tube, as before, so as to cover again the entire interior with as uniform a layer as possible. When the bulk has been poured back, the tube is stood upright, mouth down, to drain on a sheet of clean paper. In two or three minutes it will have drained sufficiently, the excess of accumulations about the mouth being wiped off on the paper now and then. The tube is then seized and rotated in a horizontal position for four or five minutes with the mouth in the draft of an electric fan, or the rotation may be somewhat longer if no air-current is available. A little experience will tell when the sack is dry enough to remove from the tube. The strong smell of ether must have somewhat subsided and the collodion must not feel wet around the mouth of the tube, as will be the case if the layer of collodion is too thick in places. If it is taken out in this condition, the thick, wet places will become clouded. The collodion is now cut free at the lips of the test-tube by means of a pin-point or other sharp instrument and the tube is filled with cool water, taking care to let it also flow between sack and wall of tube if there is any shrinkage. In a minute or two, if the work has been well done, the sack, free from air-bubbles and filled with water, may be readily lifted out of the tube. It is then placed in a jar of water, where it remains until it is ready to receive the substance to be dialyzed. These sacks are quite tough, and there is little danger of tearing them during filling and tying.

When the silicate jelly or other substance has been placed in them, the mouth is brought together and tied by means of a small rubber band, the elasticity of which keeps the sacks perfectly tight. Silicate jelly should be dialyzed for at least 12 hours, and sometimes for 24 hours, if every trace of salt must be removed. The writer fills the sacks with the silicate jelly in the afternoon and leaves them in running tap water over night. The next morning they are taken out, their contents emptied into a clean beaker, the nutrient salts added, and the fluid immediately pipetted into tubes, flasks, etc., and sterilized by heat. The nutrient substances should be dissolved in advance, so as not to delay the preparation of the medium. They should be added for this purpose to a minimum quantity of water. Some dissolve slowly, and there is a preferable order of solution, the glycerin being added last in case of Fermi's solution.

For the preparation of silicate jelly a Beaumé hydrometer for liquids heavier than water is used. C.P. hydrochloric acid of any specific gravity is diluted with distilled water until it tests 1.10° on the scale of the hydrometer when cooled

to 60° F. Clear homogeneous sodium silicate of any specific gravity is then mixed with distilled water until it is of sp. gr. 1.09° Beaumé at 60° F. A great deal of water must usually be added to the sodium silicate, and the first dilution is tedious. For example, 100 cc. of a sodium silicate of sp. gr. 1.42° required the addition of 750 cc. of distilled water to give a fluid registering 1.07 Beaumé. On adding the fluid containing the nutrient salts, and hardening, sodium silicate of sp. gr. 1.07° Beaumé gave a rather too fluid medium, and sodium silicate of much higher sp. gr. than 1.09° Beaumé is apt to set before it has properly dialyzed, or after adding the nutrient salts and before it can be tubed and slanted. Several liters of the diluted acid and sodium silicate may be conveniently made up at one time. When these are ready, equal volumes of the two are mixed. This is done by adding the sodium silicate drop by drop to the acid, rather rapidly, stirring meanwhile with a glass rod. The top part of the apparatus shown in fig. 146 may be used for this purpose. The salty, acid fluid is now ready to be placed in the collodion sacks for dialyzing in running water. It is ready for removal from the water when it is no longer acid to litmus and shows only traces of sodium chloride remaining. An exposure to the running water for 6 hours is scarcely sufficient, unless the sacks are small.

For many purposes Fermi's solution is a good one to add to the dialyzed jelly. This is made as follows, for this purpose: Freshly-boiled distilled water, 100; magnesium sulphate, 0.2; monopotassium phosphate, 1.0; ammonium phosphate, 10.0. Dissolve. Then add glycerin, 45.0.

The dialyzed silicate jelly is now poured out of the collodion sacks into a clean beaker and brought to a boil for a minute or two over an open flame (to drive off the absorbed air). It is now cooled down to 50° C. and the Fermi added. If this has been dissolved over night it must also be brought to a boil and cooled, or have the air removed under an air-pump before adding it to the silicate jelly. To 500 cc. of the dialyzed fluid, 90 cc. of the Fermi may be added. This is stirred with a clean glass rod and then quickly pipetted into test tubes.

It is now placed in the autoclave without delay in the position desired and heated for 15 minutes at 110° C. To avoid tearing the surface of the jelly by steam, the autoclave must be carefully shut steam-tight as soon as the air is driven out, and it must not be opened until the temperature has again fallen to 100° C. It is also necessary to keep the autoclave closed on account of loss of ammonia from the ammonium salt. For this reason it is desirable to dissolve the Fermi in freshly-boiled water and to pump out any absorbed air rather than to boil it out.

Other nutrient salts may be added—Uschinsky's solution, etc. The writer has had very good success with Fermi for differential purposes. Many organisms grow remarkably well on this substratum, while others do not vegetate, or make only a scanty growth.

The observations on this medium are the same as for gelatin or agar. Observe character of growth, staining of substratum (green, pink), etc.

#### SOLID VEGETABLE SUBSTANCES.

These should consist of slant cylinders in cotton-plugged test tubes half covered with distilled water and steamed 20 minutes at 100° C. on each of three consecutive

days. The addition of considerable water enables one to keep the culture under observation for several months without danger from drying out if the cotton plugs are properly made. Drier culture media may also be used. If one wishes to do so, the potato or other substance may be lifted entirely out of the water by making a constriction in the lower part of the test tube, à la Roux, or by thrusting a wad of absorbent cotton into the bottom of the test tube before the potato is introduced. The writer has not found these methods necessary. In general, I prefer vegetable media which have been sterilized in the steamer rather than in the autoclave.

The following are some of the vegetable substances recommended :

- |                   |              |               |                      |
|-------------------|--------------|---------------|----------------------|
| (1) Potato.       | (5) Turnip.  | (9) Onion.    | (13) Brazilnuts.     |
| (2) Sweet potato. | (6) Radish.  | (10) Banana.  | (14) Apple.          |
| (3) Carrot.       | (7) Salsify. | (11) Coconut. | (15) Pear or quince. |
| (4) Sugar-beet.   | (8) Parsnip. | (12) Peanuts. | (16) Pineapple.      |

These substances may be extended almost indefinitely and are very useful for making preliminary studies, inasmuch as they include many different kinds of chemical substances. The writer has used them for many years. They should be prepared with great cleanliness, especially the roots, so as to avoid including resistant spores. Sterilization is an easy and simple process if these substrata are free from spores when the steaming begins. Roots and tubers should be selected with great care, only those being taken which are sound and free from blemishes. They are now to be washed thoroughly in tap water with scrubbing and then rinsed in distilled water. With clean hands and a clean knife they are then pared, with care to remove all black specks, and thrown into a beaker of distilled, filtered or boiled water. Cylinders of the proper size may now be punched with a clean cork-borer or cut with a clean sharp knife and, after the upper part has been slanted, are thrown into another beaker of distilled water, from which they are transferred to two others before they are finally put into the tubes. It is not necessary to soak them in water over night or in antiseptic solutions. They will not brown by oxidization if they are kept under water during the early stages of preparation and are steamed as soon as they are placed in the tubes, *i. e.*, exposed to the air. They may be put into the tubes with clean fingers or by means of a pair of clean forceps.

On these different media observe the nature, amount, and rapidity of growth (always with due regard to the air-temperature, which should be recorded). Carefully determine whether there is any retardation of growth at first and, if so, to what it is due, so that more exact studies may be made subsequently in other media. Look for gas-bubbles, formation of acids and alkalies, formation of hydrogen sulphide, of crystals, of stains, of odors, destruction of starch, disappearance of the middle lamella, softening of cellulose, etc. For the first few days all cultures should be examined at least as often as once in 24 hours and, generally speaking, cultures should not be discarded until after the sixth or eighth week. These experiments should be repeated a number of times and the student should avoid drawing a hasty conclusion, since different samples of potatoes, carrots, etc., vary somewhat in composition and will at times give slightly varying results or even results which seem to be contradictory, *e. g.*, a brown pigment in some instances and not in others.

The same media, and as many other sorts as are available, should be tested raw in sterile, dry, Petri dishes 10 cm. broad and 2 to 3 cm. deep. For this purpose the vegetables are prepared as follows: First, select sound, clean specimens, especially avoiding those which are cracked open; next, scrub their surface thoroughly under the tap, and rinse them in distilled water. They are now soaked 5 or 10 minutes, or even 20 minutes, in 1:1000 water solution of mercuric chloride. They are then removed and dried with or without a preliminary rinsing in sterile water. When dry they are put on a sterile paper or plate, are cut into slices about 1.5 to 2 cm. thick with a cold sterile knife, are picked up with sterile forceps, and are put into

the Petri dishes in pairs or fours, the cover being immediately replaced. Enough of the mercuric chloride remains on the surface to inhibit the growth of any surface organisms which have not been killed outright, and experience shows that intruders are rarely dragged over the cut surface. The slices may be inoculated at once or after 36 hours incubation in a moist chamber at 30° C., or 48 hours at 25° C. The latter course is preferable. In either case, half of the slices in each dish must be kept uninoculated for

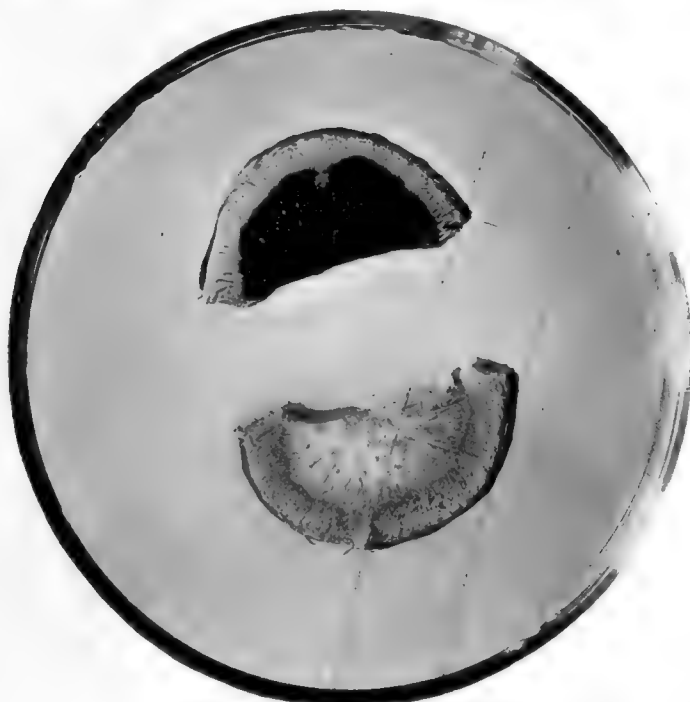


Fig. 36.\*

comparison (fig. 36). This method is well adapted to the study of various soft-rot organisms such as *Bacillus carotovorus*, *B. aroideæ*, *B. oleraceæ*, etc.

PLANT JUICES (WITH AND WITHOUT THE ADDITION OF WATER).

- |                                   |                                 |
|-----------------------------------|---------------------------------|
| (1) Juice of the host-plant.      | (4) Prune-juice.                |
| (2) Potato-broth.                 | (5) Orange-juice.               |
| (a) With sodium hydrate.          | (6) Coconut-water (unsteamed).† |
| (b) Without alkali.               | (7) Yellow corn-meal broth.     |
| (3) Cabbage or cauliflower broth. | (8) Timothy-hay infusion.       |

\*FIG. 36.—Iris-rhizome-rot organism grown on raw carrot. The check piece is unchanged, the inoculated piece has browned and softened. Incubated 4 days at about 23° C.

†This is removed directly from the nut to sterile test-tubes by means of sterile pipettes, which are useful in many ways. The pipettes should be dry-heated and kept from contamination in long, narrow, covered tin boxes. These boxes may be cylindrical or rectangular, with an end cover. The upper end of the pipette should be plugged firmly with cotton before sterilization, and this should be pushed in a short distance beyond the end, so that when the finger is placed on the end there will be an air-tight union. Scalpels, etc., should be sterilized in shorter boxes of the same kind (fig. 37).



These fluids are only a few of many that may be used. Some of them, *e.g.*, potato-broth, require special care in preparation. My own method of making potato-broth is to pass the clean pared potatoes rapidly through a grating machine and



Fig. 37.\*



Fig. 38.†

immediately throw the pulp into the required quantity of distilled water (which should be twice the weight of the potato). The beaker is now put into a water-bath and the temperature rapidly raised to 55° C. and kept there with frequent stirring for an hour. The pulp is now filtered from the fluid and the latter is immediately put into the steamer. If the steaming is long delayed the broth will be dark brown (oxidizing action of the potato-enzyme on tannins in the presence of air), and if the temperature rises much above 60° C., before the pulp is removed, some of the starch becomes gelatinous and the fluid will not filter.

All media which have boiled away to any considerable extent must, of course, be made up to the original volume or weight just prior to final sterilization.

In these culture-fluids observe the rapidity, density, and *persistency* of the clouding; whether the clouding is simple or turbid from the presence of zooglœæ; and finally, whether it is uniform in all parts of the tube. Note the character of the rim and pellicle, if any are formed, and how soon they appear; also the amount, color, and general appearance of the precipitate. The amount of the precipitate varies greatly with different media. Its quality also varies. Sometimes it consists of loose, easily separable particles; in other cases it is a viscid mass which rises as a rope-like unit when the tube is twirled (fig. 38).

Record the formation of acids, alkalies,‡ odors, gas-bubbles, stains, crystals. Does the fluid become viscid or ropy? Some organisms bring about this condition quickly in a variety of media, *e.g.*, *Bacterium pericarditidis* (*Bacillus pyocyaneus pericarditidis*), others rarely or never. Precipitates in test-tube cultures vary all the way from a scarcely perceptible trace to masses a centimeter or more in depth. Do not confound chemical precipitates with bacterial growth. Before inoculation always examine media in test-tubes for presence of slight precipitates and for contaminating organisms. In cultures of rapidly growing species, at optimum temperatures, clouding may occur in less than twenty-four hours; with slow-growing species, and

\*FIG. 37.—Tin box for holding scalpels, forceps, etc., to be sterilized by dry heat. About one-fourth actual size. A similar tin box which is very convenient for holding sterile pipettes measures 2 by 3 by 15 inches.

†FIG. 38.—Twirled culture of the olive-tubercle organism in Uschinsky's solution, showing viscidness of the precipitate in old cultures.

‡Bacterial ash is alkaline, and this ash must be carefully washed from the platinum loop in distilled water each time before it is used to transfer drops of the culture-fluid to litmus paper. The wire must, of course, be re-flamed after washing.



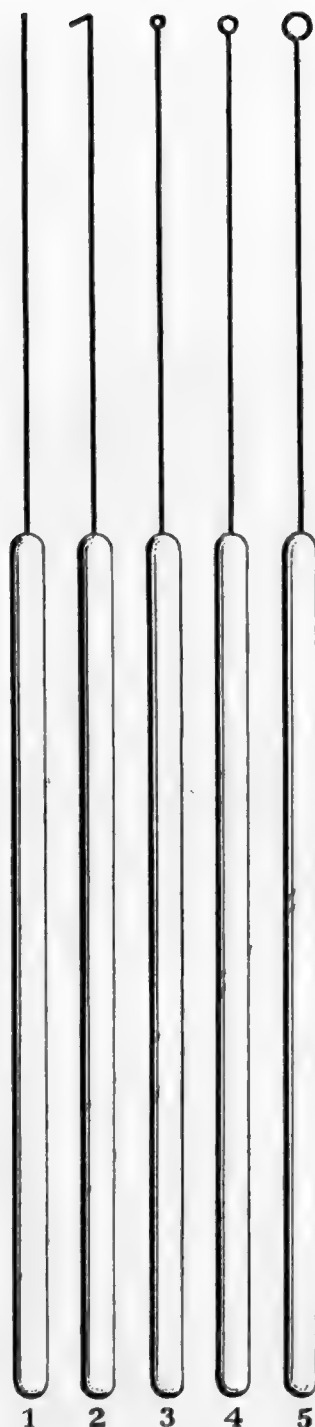


Fig. 39.†

when the medium has a retarding action, it may not occur until after two or three weeks. Of course, the rapidity of the clouding depends to a considerable extent on the size of the loop and on whether the inoculation was from a young or old, a fluid or a solid culture.

Among other tools, the student should be provided with five platinum-iridium wires set into glass handles, three of which are bent at the free end into loops of a definite size, *i. e.*, with an inside diameter of 1, 2, and 3 mm. These are made by bending around wires of the given size, and will enable one to measure out approximately uniform quantities of fluids and solids. Smaller quantities may be transferred on the extreme tip of a straight platinum needle. It is also convenient to have a platinum\* needle bent at the end into a short hook (see fig. 39). In comparing rates of growth in fluid cultures it is best to inoculate them from other fluid cultures of a given age and not from solids.

If there is any reason to think that boiling changes the nature of any of these fluids, they should be sterilized cold by forcing them through a Chamberland or Berkefeld filter. The Chamberland has the finer pores, the Berkefeld filters quicker. The simplest way of using such a filter is that first described by Dr. Theobald Smith, *viz.*, to put the fluid inside and force it out by means of clean compressed air. For this purpose select a flat-bottomed cylindrical glass vessel (a round-bottomed one is less convenient, but may be set into a hole bored in a block of wood) of a larger diameter and 5 or 10 centimeters longer than the bougie, which should be clean (previously unused), but washed by having had some liters of distilled or filtered water forced through it. Wrap the nipple-end of the filtering cylinder firmly with clean cotton for a distance of 5 or 10 cm, down. Thrust the wrapped bougie into the glass vessel securely, so that only the nipple and the cap or shoulder projects. The top of the bougie should also be wired so that it can not possibly slip down during the filtering. This apparatus should now be sterilized by putting it into the dry oven for two hours at 145° C. Wrap in clean Manila paper and heat at the same time a large cotton plug, *i. e.*, one which has been made to fit the mouth

\*Platinum-iridium is preferred to pure platinum because it bends less easily. The wire used by the writer has a diameter of 0.48 mm. The alloy as usually found on the market is said to contain about 10 per cent of iridium, sometimes less, but never more. The wire shown in fig. 39 was made to order and contains 20 per cent iridium.

†Fig. 39.—Platinum-iridium wires set into glass rods, for bacteriological work. 1, needle; 2, hook; 3, one-millimeter loop; 4, two-millimeter loop; 5, three-millimeter loop. The size of this wire is about one fifty-fifth inch.

of the cylindrical glass vessel. When sterilized and ready for use, select a piece of rubber cloth 10 or 15 cm. in diameter, cut a small slit in its center and draw it over the nipple of the bougie to protect the cotton from accidental wetting and the filtered fluid from consequent possible contamination. Now pour the fluid into the bougie (if one with a large neck has been selected this will not be difficult,

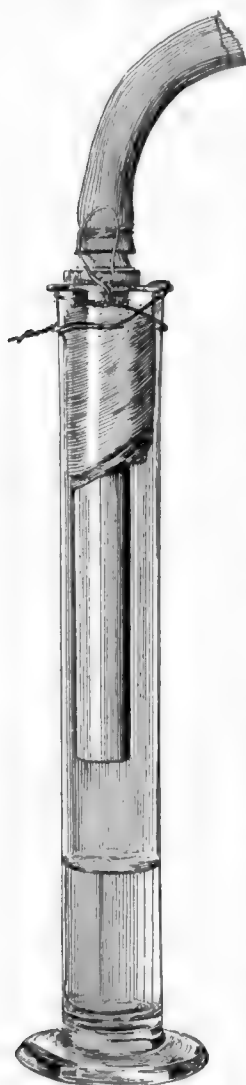


Fig. 40.\*

especially if a small funnel is used and this is kept from close contact on one side by means of a small wire, sliver, or bit of paper), and connect the nipple with the outflow-tube of the compressed-air pipe by means of an *extra-thick* rubber tube, which should be securely wired at each end, and turn on the compressed air cautiously. Fluids which are not colloidal usually filter very readily with a pressure of 15 or 20 pounds per square inch.

The filtering should always be done slowly with a minimum pressure in order to avoid the possibility of forcing small organisms through the walls of the filter. With heavy pressure this sometimes occurs when no cracks are detectable in the bougie. When the desired quantity of fluid has been filtered (fig. 40) cut off the air-blast, disconnect the tube, tilt the cylinder as much as possible, remove the bougie, and substitute the sterile cotton plug. The fluid should now be transferred *immediately*, in 5 or 10 cc. portions, to sterile cotton-plugged test-tubes by means of sterile pipettes. The removal of the bougie and the transfer of the fluid should be done in clean still air, under a hood or in a special culture-room. The tubes should not be used for several days, *i. e.*, time should be given for contaminations to show themselves, but if proper care has been exercised there should be very few contaminations or none at all. A pressure much greater than 20 pounds per square inch may be obtained by means of steam-pumps or by use of cylinders of compressed air, oxygen, or carbon dioxide, and this is sometimes necessary for colloidal substances, but should be used cautiously. These cylinders may be had from the Eagle Oxygen Company, New York. One of the most convenient filters on the market is that shown in fig. 41. It was designed by Roux and is made by Maison Wiesnegg (P. Lequeux), Paris. It is well made, very durable, quickly sterilized, and easily operated if one can command an air-blast

or other gas-pressure of 2 or 3 atmospheres.

Chamberland bougies ought not to be used continuously for more than three days. They should then be removed and baked for two hours at 145° C. (or at the

\*FIG. 40.—Simple method of obtaining small quantities of sterile fluids by means of the Chamberland filter. The other end of the rubber tube is wired securely to the outflow pipe of the compressed-air system and the fluid is forced from the inside of the filter out. This method was first described and figured by Dr. Theobald Smith. About one-fourth actual size.

temperature of an oven in which bread is baked). The reason for this lies in the fact that in three or four days time certain small organisms are able to grow through the walls of the filter and make their appearance in the filtered fluids on the other side. Persons who never bake their water-filters rest in unwarranted security. The bougies must also be handled with great care and inspected carefully after each baking for the appearance of minute cracks. To detect cracks, immerse the tube in water and blow into it. Clogged filters should be sent to the firers of china, where they may be purified by heating to dull redness.

#### ANIMAL FLUIDS.

##### BEEF-BROTH.

(a) Acid, neutral, and alkaline.

(b) The same, with addition of 0.5 per cent c. p. sodium chloride and 1 per cent peptone (Witte's peptonum siccum, Merck's brown peptone, Savory & Moore's brown peptone, etc.). This is ordinary peptonized beef-broth.

Examine as in case of plant juices. The term peptone, as it occurs in bacteriological literature, usually means commercial peptone, which is a mixture of true peptone and various proteoses or albumoses. It is therefore generally best to specify just what peptone is used. The writer now generally uses Witte's dry white peptone. Savory & Moore's brown peptone from flesh is very good for some purposes.

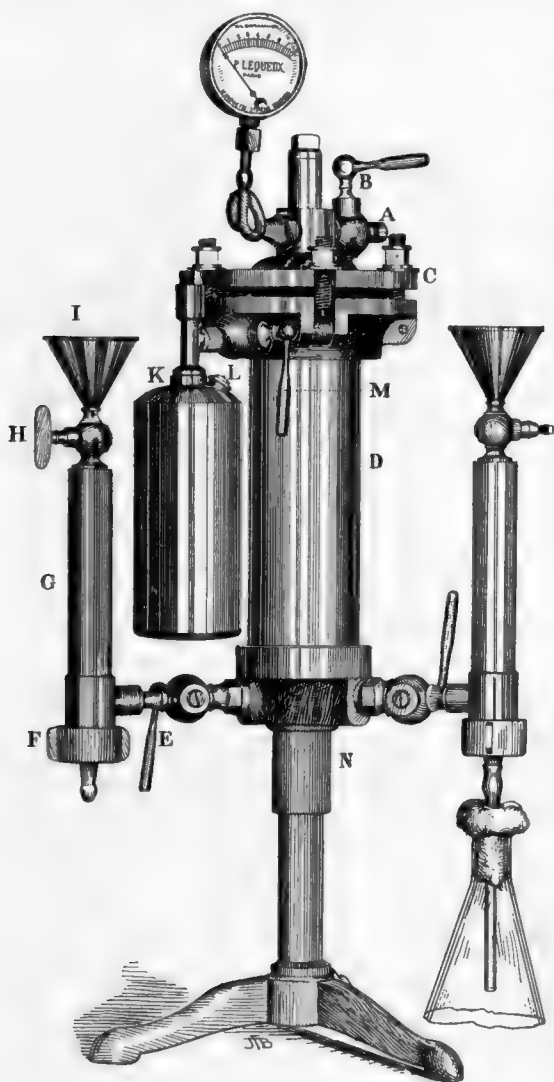


Fig. 41.\*

\*FIG. 41.—Dr. Roux's pressure-filter, made by Maison Wiesnegg (P. Lequeux), Paris. The working capacity of this filter is about 1.3 liters. The principal parts are: A, tube for connection with compressed-air system; B, cut-off; C, cover held in place by strong bolts; D, central reservoir; E, cut-off; F, screw collar which holds the bougie in place; G, heavy metal cylinder surrounding the bougie; H, cut-off, which is closed of course when the apparatus is in use; I, funnel through which G and D are filled; K, device for sterilizing the interior of the apparatus by steam under light pressure (it consists of a copper chamber partly full of distilled water, to the bottom of which the Bunsen flame is applied; the chamber may be unscrewed and removed); L, button which is unscrewed to fill the chamber with water (in its center is a steam safety valve acting under feeble pressure); M, valve which cuts the steam-generator out of the general circulation when fluids are being filtered; N, tripod-top on which the apparatus turns freely. Height, 33 inches.

*Milk.*—Milk from a clean dairy and free or nearly free from cream should be selected for use. If some cream remains it may be filtered out or removed by the centrifuge (fig. 43). The milk should not be acid to the taste and should not contain formaldehyd or other antiseptic substances which milk-dealers sometimes add to dirty milk to improve its keeping qualities. It should be steamed in wire-crates 15 minutes at  $100^{\circ}$  C. on each of four consecutive days (10 cc. portions in test-tubes), and should not be used until at least a week after the last steaming. Such milk should titrate + 12 to + 17 or thereabouts with sodium hydrate and phenolphthalein. Milk-cultures should be kept under observation at least six or eight weeks.

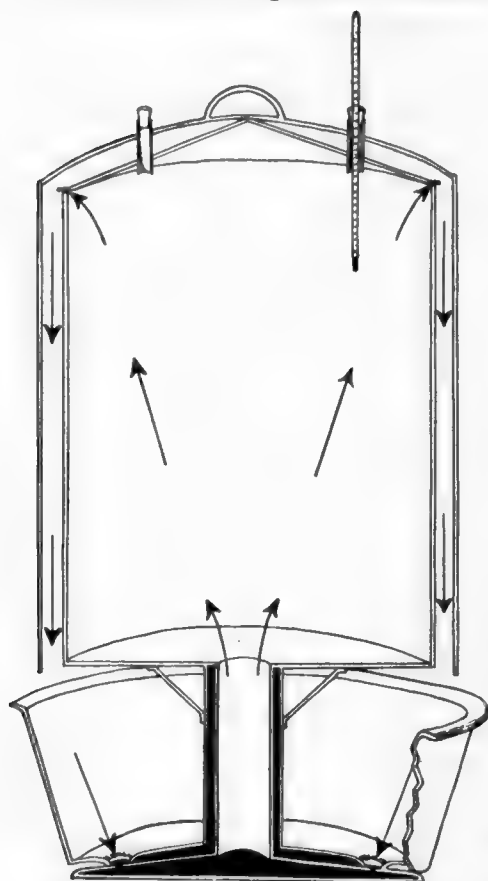


Fig. 42.\*

sometimes, especially in dirty milk, and the milk is then difficult to sterilize.

Several experiments made by the writer with milk from Washington dairies have shown that Franz Lafar's statement in *Technische Mykologie*, Bd. I, p. 189, while probably true for the milks which he tested, is not true when stated as a general proposition. In brief, this statement is that nine out of ten milks are not

Observe in particular:

- (a) Separation of the casein without the development of any acid, indicating the presence of the lab, or rennet, ferment. The milk usually becomes more alkaline.
- (b) Saponification of the fat. The fluid becomes transparent without any precipitation of casein; but the caseinogen may be thrown down subsequently by acidifying the clear liquid.
- (c) Ropiness. The fluid becomes viscid, and strings when touched. This viscosity is sometimes so great that an entire pail of milk may be inverted without immediate loss of its contents. See striking figures in Ward's papers ('99 and '01, Bibliog., XLVII).
- (d) Formation of acids. This occurs with or without evolution of gas, and usually with the final separation of the whey from the casein at room temperatures or on boiling. Boil if necessary.
- (e) Re-solution of precipitated casein (trypsin ferment); formation of crystals (tyrosin, leucin, etc.).
- (f) Gelatinization of old cultures. Milk alkaline.
- (g) Changes in smell, color, and taste.

In using milk it should not be forgotten that anaerobes are sometimes present (Theobald Smith) and also organisms of the dunghill which will grow only at temperatures above  $40^{\circ}$  C. Very resistant spores of aerobic species, growing at temperatures below  $40^{\circ}$  C., are present also

\*FIG. 42.—Section of Arnold steam sterilizer. Water enters the double bottom through a few small openings indicated by two arrows in the water-pan. The other arrows show movement of the steam. In this form the outer jacket (of copper) is lifted off to put in or remove media.

sterilized by steaming twenty to thirty minutes on three consecutive days, but will develop bacterial growths when put into the thermostat. If such were really the case, milk would be one of the worst of culture-media instead of one of the best. The general experience of bacteriologists is not in accord with this statement. Occasionally, in my own experience, a single steaming of five or ten minutes has

sufficed to sterilize milk completely, at least so far as relates to organisms which grow aerobically and at temperatures under  $40^{\circ}\text{C}$ . Such milks have remained unchanged for two or three months at room temperatures ( $20^{\circ}$  to  $25^{\circ}\text{C}$ .), and also in the thermostat at blood heat. For anaerobes, or organisms which will grow only at temperatures above  $40^{\circ}\text{C}$ ., I have not tested.

One possible source of error in the use of steam for sterilization is ignorance of the exact temperature of the steam-chamber. Every steam-sterilizer should have a hole punched through the top, into which is fitted a cork through which a thermometer projects into the chamber. In this way may be determined beyond doubt for just how many minutes the media has been exposed to steam at  $100^{\circ}\text{C}$ . The Arnold steam-sterilizer, which is one of the best,<sup>†</sup> is greatly improved by this simple device (fig. 42 and



Fig 43.\*

pl. 6). In this sterilizer there is a double bottom under the water-pan. The lower bottom is in contact with the Bunsen flame. Through small holes in the upper

\*FIG. 43.—Improved Lautenschläger centrifuge. Capacity, 540 cc. Revolutions per minute, 3,000 to 4,000. It requires about 3 horsepower to run the apparatus at this high speed. About one-eleventh natural size.

†This remark does not apply to the Arnold combination steamer and dry oven, which can not be recommended.

bottom the water drips to the lower bottom and is quickly converted into steam which streams through a central chimney into the bottom of the sterilizing chamber. The latter has two walls, with a considerable air-space between, open at the bottom. The streaming steam passes over the top of the inner wall downward into this air-space and escapes into the pan as condensation water. Theoretically this is a very

perfect sterilizer, and it is so in practice when new, but not infrequently it leaks, and sometimes the openings in the upper bottom are too large or become clogged by mud. When in perfect working order it takes only a few minutes to get a temperature of 100° C.

Tubes should always be steamed in wire-crates (fig. 44) so that the streaming steam may have full access to all parts. Tubes of media steamed in cans or beakers often spoil. They seem to retain a cushion of air about them which interferes with the action of the steam.

*Litmus milk.*—Litmus milk of a good quality may be made by dissolving Merck's dry, lime-free c. p. blue litmus to saturation in distilled water

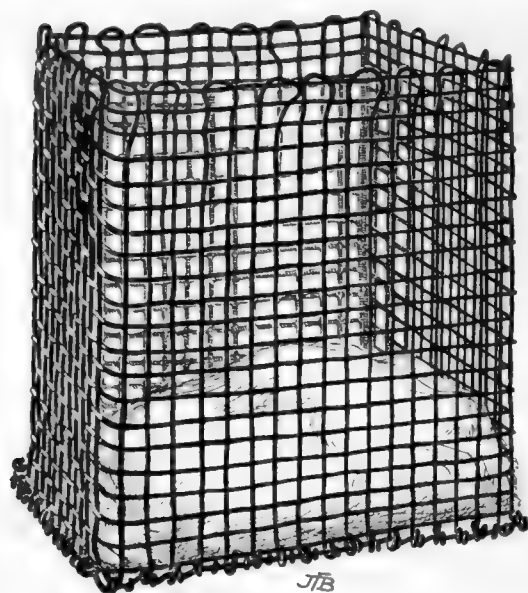


Fig. 44.\*

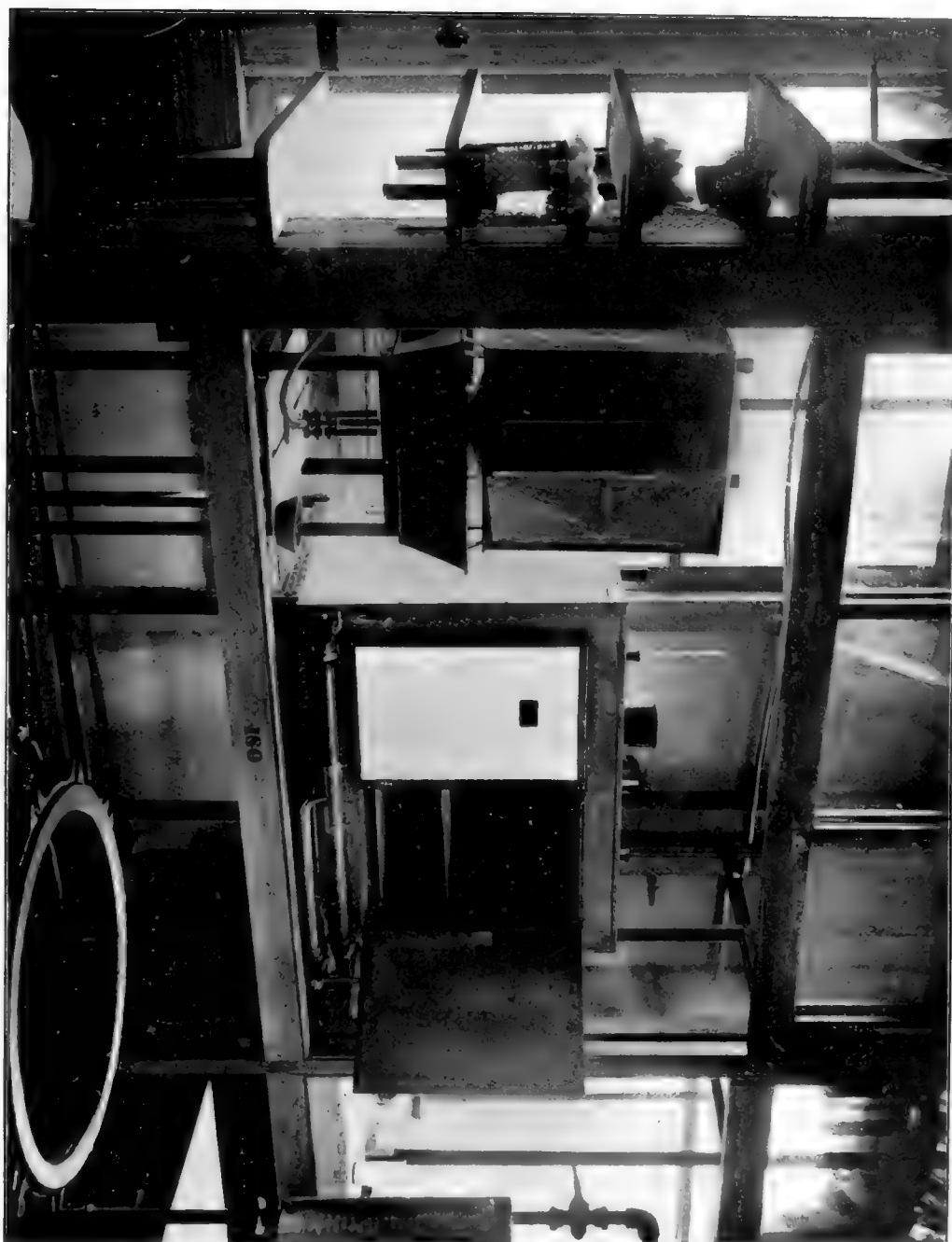
(1:15) and then adding one part of this blue fluid to each fifty parts of milk. The milk should be a deep lavender color. Much inferior litmus is on the market. Large use should be made of this fluid. In addition to observations under "Milk," note how rapidly the litmus reddens, blues, or becomes reduced, and how soon the color returns. Will it return at once on steaming the culture?

*Rice cooked in milk.*—(One or two grams to 10 cc. in each test-tube). This is useful for study of some chromogens.

*Loeffler's solidified blood-serum.*—Observations under this and the following heads are the same as for gelatin slant cultures. The plant bacteriologist must in general obtain blood-serum from the animal bacteriologist. The solidified serum may also be used plain, *i. e.*, without the addition of grape-sugar.

*Egg-albumen.*—This is solidified and used in the same way as blood-serum. The end of the egg from which the albumen is poured must be thoroughly flamed before it is broken, and care must be used in the transfer to test-tubes so as to exclude air-borne germs as far as possible, otherwise the sterilization will be difficult. The albumen of eggs may be cut with sterile scissors.

\*FIG. 44.—Wire-crate for holding tubed culture-media which is to be steamed. About two-fifths actual size. A tuft of cotton on the bottom prevents the breaking of tubes.



Hood under which apparatus and culture-media are sterilized.  
At the left is the Arnold steam sterilizer (Boston Board of Health pattern). At the right is the large size Lautenschläger dry hot-air sterilizer. In front, autoclave with cover removed.





*Egg-yolk.*—This is poured into test-tubes and solidified in a slanting position by heat (80° C.), or the egg may be boiled hard and the yolk cut with a sharp knife and transferred to sterile Petri dishes. If desired, the yolk and white may be mixed before solidifying, *i. e.*, by shaking the egg vigorously before breaking the shell.

#### SYNTHETIC MEDIA AND OTHER SPECIAL MEDIA.

The student should try the following media. He should also invent media to suit special cases. The kinds of media I have in mind are the opposite of universal,



Fig. 45.\*

to-wit, such as will favor the growth of some organisms while preventing that of others. The acid phosphates and many other substances are useful for this purpose. The field is comparatively new, and much is to be learned by careful experimenting.

1. Dunham's solution.
2. Peptone-water (1 or 2 per cent) with addition of various carbohydrates, acids, etc.
3. Sugar-free beef bouillon with Witte's peptonum siccum (for the indol test).
4. Cohn's solution.
5. Uschinsky's solution.

\*Fig. 45.—Oven for solidifying and sterilizing blood-serum, nutrient starch-jelly, silicate-jelly, etc., at temperatures below 100° C. When in use the temperature is controlled by means of a Tollen's thermo-regulator (see fig. 35).

6. Uschinsky's solution with various carbon compounds substituted for the glycerin (fermentation tubes).

7. Fraenkel and Voge's solution.

8. Raulin's solution.

9. Fermi's solution.

10. Water (distilled), 1,000,000 mg.; dipotassium phosphate, 2,000 mg.; ammonium phosphate, 100 mg.; magnesium sulphate, 100 mg.; sodium acetate, 5,000 mg.

11. Same, with the carbon compound changed, *e. g.*, with sodium formate substituted for sodium acetate. Sodium formate and phenolphthalein may be added also to bouillon or agar (2 per cent) for observations during the early stages of growth, some organisms reddening this medium promptly by decomposition of the sodium salt. (See a recent paper by Omélianski).

12. Nutrient starch-jelly for study of diastasic action. (See Proc. Am. Asso. Adv. Sci., 1898, p. 411, or Centralb. f. Bakt., 2te Abt., Bd. V., p. 102.)

One gram of starch is rubbed up with a sterile glass rod in 10 cc. of the sterile nutrient fluid (Uschinsky's solution, etc.), placed in a slanting position, in test-tubes, and solidified in a blood-serum oven (fig. 45) or in the top of a steamer with the vents left open. There should be several heatings of two hours each to insure sterilization. The temperature should not exceed 93° C. nor fall much below 85° C. Sterilization is rendered much easier if the starch is prepared in a cleanly way. The only difficulty the writer has experienced is in the formation of a thin film of semi-opaque solidified starch on the walls of the tubes above the slant. This often cracks off, however, during the heatings, and is largely obviated by placing the tubes in a slanting position before the starch is rubbed up in the fluid, taking care to soil the walls above the slant surface as little as possible during the operation. The potato-starch is prepared as follows:

One-half bushel of large smooth potatoes are scrubbed, and the black specks dug out; they are then soaked for 45 minutes in 1:1000 mercuric-chloride water. Meanwhile the hands are scrubbed clean and given a five minutes washing in the mercuric-chloride water. The tubers are now rinsed in sterile water, pared deeply, grated as for potato-broth, and thrown into beakers containing several liters of distilled water, where the pulp is worked over with the hands to liberate as much starch as possible. The starchy water is now removed from the pulp by passing it through several folds of surgeon's gauze, squeezing out of the pulp as much of the fluid as possible. When the starch has settled the brownish fluid and floating fragments are poured off or decanted, and fresh distilled water is added. The smaller fragments of cell-wall, etc., are then removed by forcing the starch (stirred up in water) through a moderately fine-meshed towel (not too fine) with gentle hand-rubbing, into another beaker. Most of the medium-sized and finer starch-grains pass through, leaving in the towel the coarser grains and those fragments of cell-wall which passed through the coarser meshes of the surgeon's gauze. The purified starch is now allowed to stand for about a week in the ice-box in distilled water (3 liters or more per beaker or jar). The water is siphoned off twice a day at first, and afterwards once a day, the starch being stirred up thoroughly every time fresh water is added. Finally the starch is drained very free from water, scooped out with sterile spoons or spatulas, placed in uncovered sterile Petri dishes, and dried in the blood-serum oven at 56° C., the cover being raised an inch (on corks) to let the moisture out. One-half bushel of sound potatoes should yield from 400 to 500 grams of air-dry aseptic starch.

Potato starch has been selected because it is easy to prepare, but other starches might yield interesting results. Bacteriologists now pay great attention to the fermentation of sugars, but thus far very little consideration has been given to the action of bacteria on starches and celluloses. Whatever starches are used, they should be prepared in the laboratory, under aseptic conditions, so as to exclude spore-bearing organisms.

13. Starch-jelly with addition of various sugars, gums, and alcohols (for study of organisms having little or no action on starch).

14. Tubes of slant nutrient agar (+ 15 of Fuller's scale) with varying amounts of *c. p.* glycerin, 2 to 10 per cent or more.

15. Tubes of 10 cc. slant agar with 10, 20, and 30 grams of grape-sugar.
16. The same, with the same amounts of cane-sugar.
17. Gelatin with cane-sugar, varying amounts.
18. Gelatin with malic acid. (17 and 18 may be combined.)
19. Gelatin plates with soluble starch and 1 per cent potassium iodide and with or without 1 per cent potassium nitrate. Try a mixture of the pear-blight organism and *B. coli*. Can the colonies be distinguished in this way using the nitrate?
20. Agar plates with various sugars and the addition of calcium carbonate, or zinc carbonate, for detection of acid-forming colonies. ('91, Beyerinck, Bibliog., XX.)
21. Silicate-jelly. See p. 36. Known also as silica-jelly.
22. Nitrate bouillon (+ 15 bouillon with 1 per cent potassium nitrate).
23. Triple-distilled water and nutrient mineral substances free from nitrogen. The same, with addition of potassium nitrate. The same, with other nitrogen foods, *e. g.*, sodium asparaginate.
24. Bouillon with lead acetate.
25. Bouillon with neutral red.
26. Salt bouillon, *i. e.*, + 15 bouillon with varying amounts of c. p. sodium chloride (1 to 5 per cent).
27. Standard peptonized bouillon with varying amounts of sodium hydrate (from + 40 to — 40) for determining the optimum reaction and the tolerated range of acidity and alkalinity.

Synthetic media may be varied indefinitely to fit special cases and are often extremely useful as differential tests. They have frequently been condemned because some particular organism has not grown *well* in them. The very fact of feeble growth or of no growth is, however, a matter of interest, and not infrequently a means of distinguishing organisms which resemble each other in many particulars. The value of such media becomes apparent at once when a number of organisms are compared. Synthetic media afford more exact methods of research than do the common media, and their value must increase rather than diminish as time goes on. (Consult Grimbert in Archives de Parasitologie, T. I, pp. 191-216.) It does not follow, however, that the common media should be at once abandoned. *Festina lente* is a good rule. The formulæ for some synthetic media are given under "Formulæ." For others see various text-books and the papers cited in the Bibliography under XVI, XVII, XVIII, XXV, etc.

#### RELATION TO FREE OXYGEN.

(1) *Surface and deep growths.*—Note the behavior of deep stabs in tubes of recently steamed gelatin and agar, or of the colonies in shake-cultures of gelatin and agar which are protected from the free action of air by pouring into the tubes as soon as solidified another tube of gelatin or agar in the surface layers of which, as an additional precaution, some active aerobe may be grown, *e. g.*, *Bacillus subtilis*. Observe also the relative rate of growth of buried and surface colonies in plate cultures, growth under sterile mica plates, etc. Of course, whether an organism will or will not grow under the conditions mentioned depends often to a large extent on the composition of the culture medium. It might be able to respire in the presence of grape-sugar or cane-sugar, but not when milk-sugar or glycerole is substi-

tuted. It will not do to conclude that an organism is a strict aerobe until it has been tested anaerobically in the presence of a variety of carbon foods with uniformly negative results. One who has had some experience may often give a shrewd guess as to behavior in fermentation-tubes by carefully noting the growth of buried and surface colonies in ordinary media.

(2) *Fermentation-tubes*.—The fluids may be Uschinsky's solution (without the glycerin unless this is the carbon compound to be tested); peptone water (2 per cent Witte's peptone with 0.5 per cent sodium chloride); and filtered tap water, or sugar-free beef bouillon with addition of 1 per cent Witte's peptone (preferably for most purposes this latter fluid). The substances to be tested (which should be chemically pure or as nearly so as possible) are grape-sugar, fruit-sugar, cane-sugar, milk-sugar, galactose, maltose, dextrin,\* mannit, dulcit, raffinose, glycerin, ethyl alcohol,† methyl alcohol, acetone, ammonium lactate, ammonium tartrate, asparagin, sodium asparaginate, urea, etc. One to 5 per cent of the various sugars, etc., may be used; 2 per cent is a good quantity.

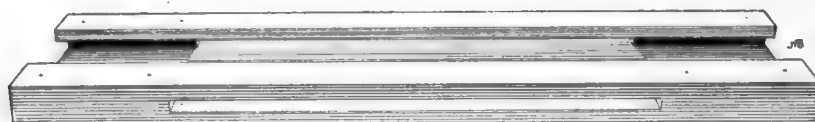


Fig. 46.†

Observe carefully what substances induce clouding in the closed end and whether any gas is produced. Test from time to time for acids. The relative vigor of growth in the open end should also be noted. Does growth stop in the U with a sharp line of demarcation? Does the addition of calcium carbonate reduce or prevent the formation of gas or favor growth in any way? Is the reaction in the closed end, as the result of growth, different from that in the open end? Pipette out all the fluid from the open end, determine its reaction to litmus, and then test the reaction of that which remains. How is the difference, if any, accounted for? If growth finally ensues in the closed end, is there any reason for thinking it due to absorbed air? How can this be determined?

It should be remembered that often, after a time, air is absorbed into the closed end of fermentation-tubes and may lead to confusing results. For this reason, if they have stood on the shelf any length of time after sterilization, they should be re-steamed and the bubble of air tilted out before they are inoculated. They

\*The dextrin should be freely soluble in cold water and should not give any red reaction with iodine—i. e., should be free from amylo-dextrin (erythro-dextrin). Such dextrin is hard to procure.

†This and the next four should be added, after sterilization, by means of sterile pipettes. The ammonium salts may be obtained in a sterile condition without loss of ammonia by dissolving 10 grams in 200 cc. of water and forcing this through a Chamberland filter into a sterile flask, from which the proper quantity may be pipetted into the culture medium after sterilization.

‡Fig. 46.—Wooden carrier for fermentation-tubes, the flanging base being held under the grooves. Much reduced.

should be disturbed as little as possible after inoculation, and especially all tiltings or rough jarring should be avoided. They may be carried in a wooden rack (fig. 46). All culture-media, whether inoculated or not, should be protected from light.

Figs. 47, 48, 49 show fermentation-tubes in actual use.

The pattern of fermentation-tube preferred by the writer is that slight modification of Einhorn's tube designed by Dr. Theobald Smith (see Wilder Quarter Century Book). The tubes may be had from Emil Greiner, New York. Certain

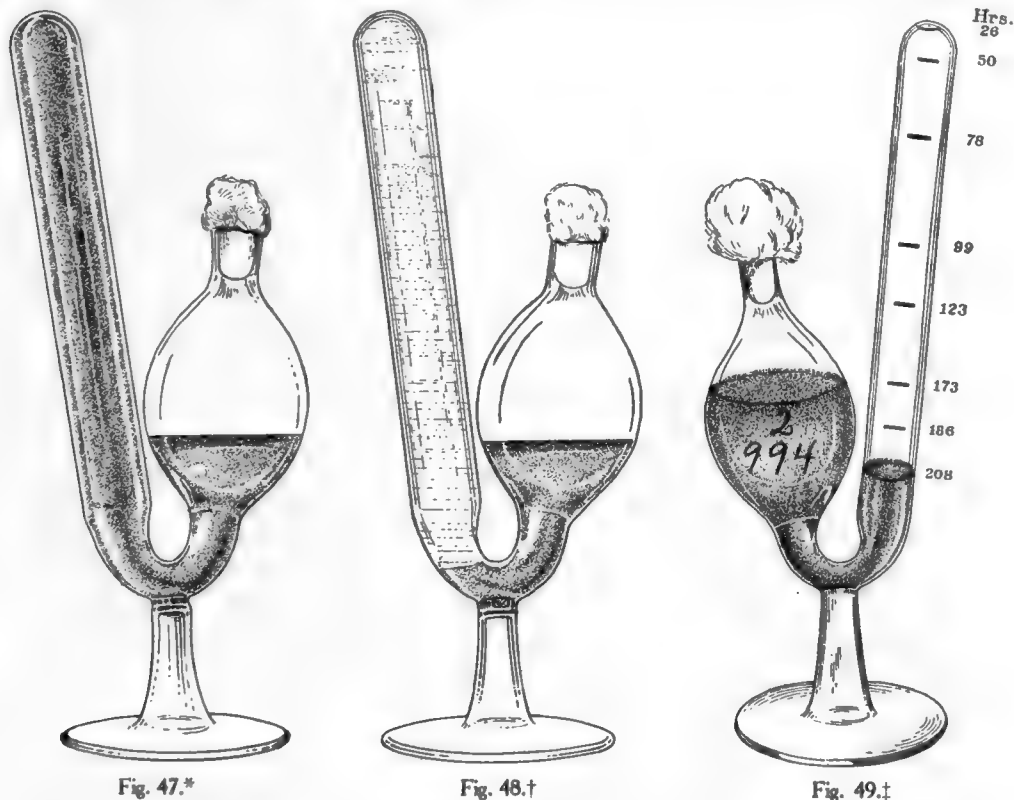


Fig. 47.\*

Fig. 48.†

Fig. 49.‡

forms of tubes should not be used. One of these, a short, thick tube with a wide U, in use in some laboratories in this country, allows air to pass readily into the closed end and is entirely worthless. A sample tube of this sort was filled with

\*FIG. 47.—Fermentation-tube with *Bacillus tracheiphilus*, showing absence of gas and uniform clouding in open and closed end in the presence of grape-sugar. The fluid consisted of water, 400; Savory & Moore's peptone, 4; sodium chloride, 1; c. p. grape-sugar, 2; saturated solution carbonate of soda (20° C.), 20 drops, i. e., enough to render the fluid slightly alkaline to litmus.

†FIG. 48.—Fermentation-tube with *Bacillus tracheiphilus*, showing inability of organism to grow anaerobically with glycerin as the carbon food. Fluid, distilled water with 1 per cent Witte's peptonum siccum and 1 per cent Schering's c. p. glycerin. Copious growth in open end and in outer part of U; none in the closed end.

‡FIG. 49.—Fermentation-tube of cane-sugar peptone water inoculated with a white, gas-forming organism plated from a spot disease of sisal hemp. The total amount of gas produced and its rate of evolution at 20° to 23° C. are indicated by marks on the closed end of the tube.

beef-bouillon and steamed every twenty-four hours for seven or eight days, a large bubble being tilted out each time and appearing just as regularly during the next steaming. Naturally, no strict anaerobe would grow in such a tube and every aerobe would appear to be a facultative anaerobe. The neck of the fermentation-tube should be as narrow as consistent with filling and cleaning. All wide-necked tubes should be discarded. The behavior of the closed end with reference to the

absorption of air may be tested by adding litmus-water and 5 per cent grape-sugar to the bouillon. On steaming, the litmus is reduced. If there is no air in the closed end the litmus remains reduced, while in the open end exposed to the air it soon oxidizes back to its original color.

Other things to be observed are :

- (3) *Growth in hydrogen.*
- (4) *Growth in carbon dioxide.*
- (5) *Growth in vacuo, various degrees of exhaustion.*
- (6) *Growth in vacuo, remnant of oxygen absorbed by the mixture of caustic potash and pyrogallol (same as pyrogallic acid).*
- (7) *Growth in nitrogen (air with the oxygen absorbed, normal air-pressure).*

The hydrogen and carbon dioxide, which are required in considerable quantities, may be generated in Kipp gas-generators. There is a choice in generators. The writer has not found any kind which is entirely satisfactory. The one which has given the least trouble is shown in fig. 50. The objection to this generator is the large volume of dead acid which soon accumulates at the bottom. The accumulation of dead acid is entirely obviated in the de Koninck generator, but the writer has only recently obtained this apparatus and has not yet had enough experience with it to speak unqualifiedly. It furnishes a large amount of gas and its generation may be stopped very quickly, but the acid chamber is inconveniently bulky (10 liters) and in case of breakage a destructive flood

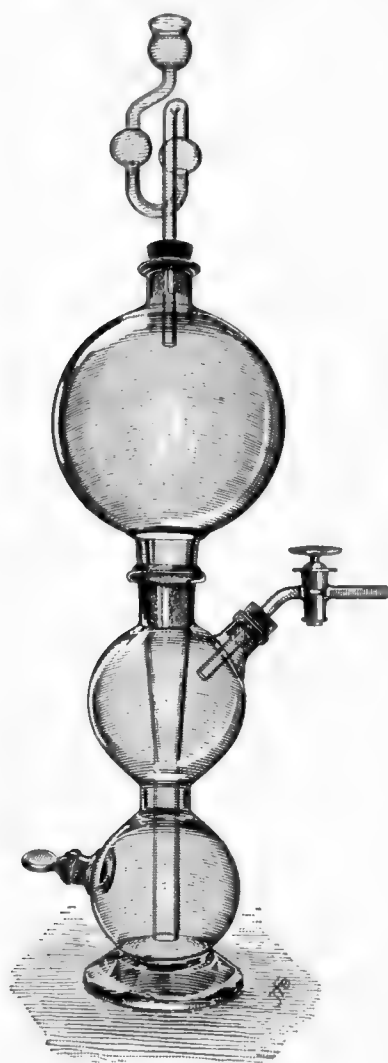


Fig. 50.\*

would be poured out into the laboratory. To avoid this the apparatus should be set into a deep enameled iron pan. The action of the apparatus depends on the fact

\*FIG. 50.—Kipp gas-generator for making carbon dioxide or hydrogen. When not in use the pressure of the gas forces the acid off the marble or zinc (in the middle compartment) and stops its evolution. Much reduced.

that acid on which zinc has reacted has a greater specific gravity than unused acid and diffuses downward through the whole fluid when it is forced back from the zinc-chamber into the top of the acid-tank.

Another form of hydrogen generator is shown on plate 7. When in use the lower bulb is filled with acid and also the stem of the upper one. This gives a sufficient column of liquid to force the gas through the five wash-bottles. All the joints should be coated with Darwin's wax-mixture, set together firmly, and wired in place. Excessive liberation of hydrogen sulphide is avoided by standing the generator in ice water. The ruler is 12 inches long. The same style of apparatus may be used for the generation of carbon dioxide.

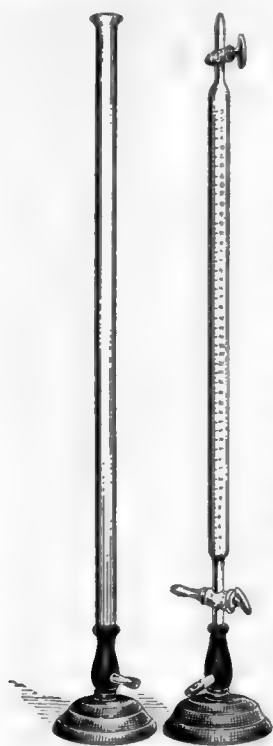


Fig. 51.\*

These gases must, of course, be carefully washed to remove accidental poisonous impurities, by passing them through wash-bottles containing various solutions. For the carbon dioxide, which is usually generated from c.p. hydrochloric acid, diluted with twice its volume of boiled water, and marble chips (which should be boiled in advance), it is sufficient for many purposes to pass it through strong solutions of sodium hydrate (10 per cent), potassium permanganate (10 per cent), and water, arranged in the order indicated. Most of the oxygen may be removed by passing through three wash-bottles containing a mixture of pyrogallol and strong caustic-potash water or caustic-soda water (10 per cent). When in use the stopcock between the generator and the first wash-bottle must not be cut off, otherwise the small amount of carbon dioxide in the wash-bottle will soon be absorbed by the soda and fluids will be forced over (backward) from the other bottles by inequalities in the gas-pressure. The place to cut off the gas-flow is close to the Novy jar or other receptacle.

For testing the purity of the gas, *i. e.*, its freedom from air, 100 cc. may be drawn off into a Hempel burette (fig. 51), equalized with the air-pressure and run into the simple Hempel pipette for liquid reagents (fig. 52), the bulb of which is filled with strong potash water (2 water + 1 potassium hydroxide). If any gas remains after thorough exposure to the potash, it may be measured by passing it back into the burette. One should get with the pipette an *iron* stand and about 2 yards of capillary glass tubing.

The scrap-zinc used for generating the hydrogen should contain some lead, but should be free from arsenic, antimony, and phosphorus, and the sulphuric acid should be chemically pure. For use the acid is diluted largely with water (1 : 9). Hydrogen generated with zinc, especially if the evolution is rapid so that the solution is warmed, contains considerable hydrogen sulphide and may contain phosphureted

\*FIG. 51.—Hempel's burettes for gas-analysis. Height, 25 inches.



hydrogen or arseniureted hydrogen; it should therefore be passed not too rapidly through the following solutions in the order indicated: Saturated solution of lead acetate, 5 per cent solution of silver nitrate, 10 per cent potassium permanganate, 10 per cent sodium hydrate containing pyrogallol, distilled water. When ready for use the purity of the hydrogen may be tested by burning in test-tubes (mouth

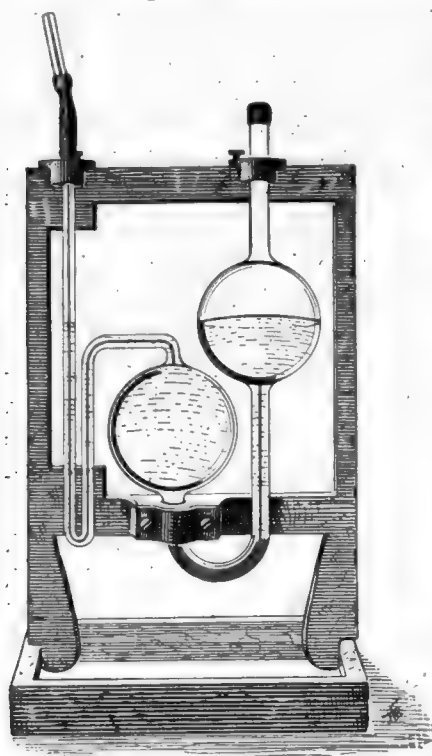


Fig. 52.\*

down, and also, if necessary, by the ordinary methods of gas-analysis. To avoid the evolution of hydrogen sulphide the generator may be plunged into a jar of ice-water, as shown in plate 7. Special care must be taken in sealing jars containing hydrogen, otherwise it will escape. In use, the gas is allowed to bubble slowly through the fluids into the culture-chamber, a large well-clamped Novy jar, the other tubular opening of which is connected air-tight with the tube of the vacuum pipe. The jar is first pumped out and the hydrogen is then allowed to enter. When the jar is full, the glass stopcock nearest it (at the left in plate 7) is turned, and then, after allowing a few minutes for diffusion, the mixture of air and gas is pumped out. The vacuum cock is then turned off and the hydrogen is again turned on slowly. This process is repeated five or six times, the gas being passed into the jar very slowly the last two times, so that it may be washed very clean. The Novy jar is then sealed, disconnected, and set away in the dark.

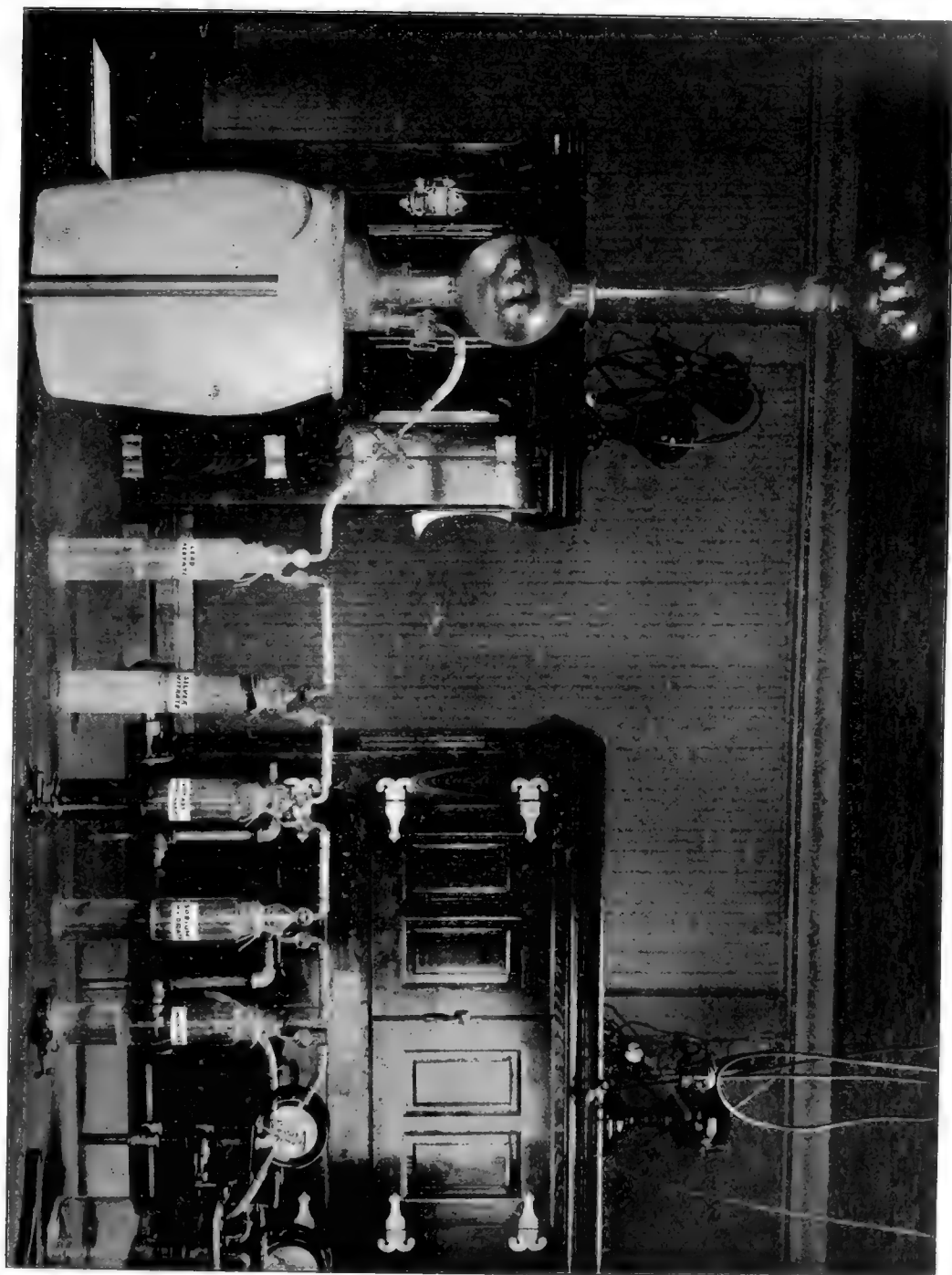
The gas must, of course, enter each wash-bottle through the long stem. It is desirable to have each wash-bottle two-thirds full of fluid, and there must be no leaks in any part of the apparatus.† The hydrogen should be cut off before each exhaustion of the jar by turning the stop-cock nearest the jar. The cock also should be turned off before sealing glass tubes with flame and it must, of course, be known that the gas is free from admixture with air, otherwise an explosion will occur.

It is easier to keep air out of gases than to remove it. The greatest care should therefore be taken to drive it out of a culture medium before it is inoculated. For the same reason gas should be allowed to flow for some time before it is collected so as to displace air which may have diffused into the generator and wash-bottles. This is also the reason why the water which is used to dilute the acid and the marble chips should be boiled. If there is much air mixed with the gas it is not at all likely that a single wash-bottle of sodium hydroxide and pyrogallol,

\*FIG. 52.—Hempel's simple pipette for liquid reagents used in gas-analysis. Breadth of stand, 7 inches.

†Consult a paper by Ewell, *Centralb. f. Bakt.*, 2 Abt., III Bd., p. 188.





Hydrogen generator with wash-bottles.

Back of the table are the cool boxes containing stock-cultures of various organisms; between the two is a gelatin box; at the extreme left is one corner of a large ice-box.



or even two or three in series, will completely remove it, since the bubbles of gas are in contact with the fluid only at their surface and for a very brief time. Hydrogen must be passed through 5 wash-bottles of sodium hydroxide and pyrogallol if every trace of oxygen is to be removed. From nitrogen or carbon dioxide the last traces of oxygen may be removed by passing it over copper filings inclosed in a piece of gas-pipe which is heated red hot in a small furnace containing about 20 Bunsen flames in series. The gas-pipe may be 0.75 inch in diameter and about 3 feet long, plugged at the ends with tight-fitting rubber stoppers, the middle 2 feet filled with the copper fragments. The gas should be allowed to flow only in rapid bubbles, not in a stream (Dr. Day).

The test-tube cultures may be placed in Novy jars, securely waxed (fig. 53), or in large, thick-walled test-tubes made impervious with sealing wax (see Sternberg, Manual, fig. 53; Text-Book, fig. 53). Media designed for use in any of these gases should be resteamed immediately before inoculation, and if one is experimenting with unknown or with very sensitive anaerobes the boiled media should be allowed to cool in an atmosphere of hydrogen. Francis Darwin's wax-mixture has been found useful for luting.

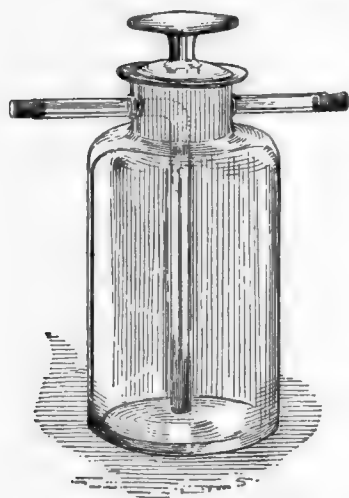


Fig. 53.\*

When large Novy jars are used (fig. 54), the thoroughly waxed gaskets must be clamped down securely and tested for leaks by preliminary exhaustions. If any are discovered, additional wax must be used and the clamps must be screwed tighter. To determine whether there is any subsequent entrance of air it is always best to include along with the cultures one or more tubes containing some sub-

stance which is reduced in the absence of free oxygen, but which readily oxidizes to some different color as soon as traces of air are mixed with the gas in the jar. Methylene blue in recently steamed bouillon or gelatin with 5 per cent grape-sugar is one of the best pigments for this purpose. In the absence of free oxygen it becomes a colorless substance; with the entrance of traces of air it becomes blue. Usually, however, the fluid or solid holds on to a trace of color at its surface. A solution of bilirubin is also said to be very sensitive to free oxygen and a good test for its presence.

Some care is necessary in order to avoid erroneous conclusions when pyrogallol and caustic potash are used to absorb the oxygen. The vessel must not leak, enough of the mixture must be used to absorb all the oxygen, and the action must be rapid enough so that the oxygen will have been removed completely before visible growth of the organism can possibly have taken place. Neglect of these

\*FIG. 53.—Novy jar. Small size (wide mouth) for test-tube cultures. Only those with mouths at least  $2\frac{1}{2}$  inches wide are serviceable. Height to mouth of jar,  $7\frac{1}{4}$  inches.

precautions has led to the statement that certain strict aerobes are able to grow on ordinary media in the absence of oxygen, and that anaerobes are very uncertain in their behavior on standard media. Old pyrogallic acid should be avoided and some preliminary experiments should be made as to the rapidity of the absorption of the

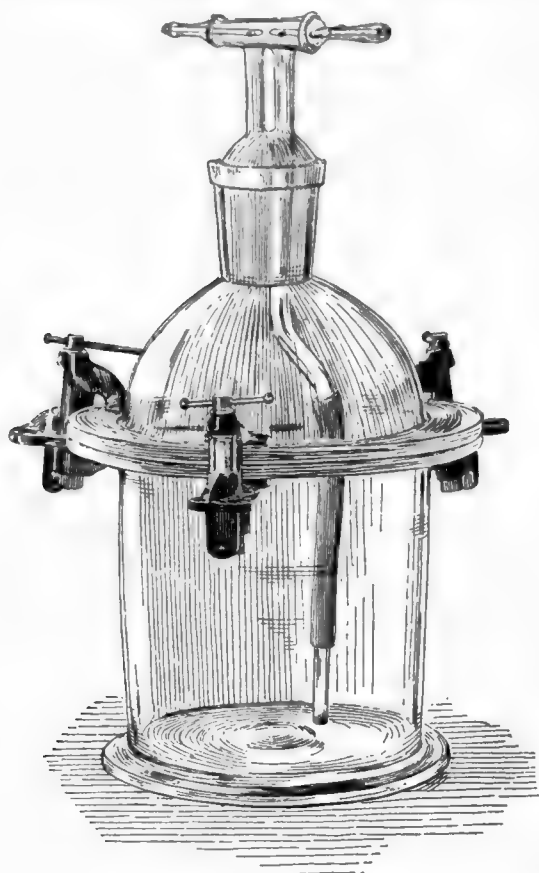


Fig. 54.\*

oxygen from a given space before the organism is tested. The writer found one brand of pyrogallol which removed the oxygen from a small space in six hours, another required about eighteen hours, a third required several days (time enough for a strictly aerobic organism to make a visible growth). Leaks may be detected readily by including with the cultures a fermentation-tube, the inclosed arm filled with water except for a small bubble of air. On absorption of the oxygen this bubble expands to a diameter which should remain constant if the jar continues air-tight.

The gas remaining in receptacles from which the oxygen has been removed by the potash-pyrogallol method is not pure nitrogen, but nitrogen plus a variable small amount of carbon monoxide, which is said to be most abundant when the oxygen is absorbed slowly. This small amount of CO is harmless to many bacteria, but the writer has some reason for suspecting that it is inju-

rious to others, even if it does not entirely inhibit growth.

The writer has found the following contrivance (fig. 55) a very simple one for testing the ability of organisms to grow in nitrogen: A U-tube of thick, clear glass, with arms about 10 to 12 inches long, open at the ends and having a uniform inside diameter of about 1 inch, serves as the culture-chamber and gas-receptacle. Two short, rimless, cotton-plugged test-tubes containing the media to be tested are inoculated and thrust one above the other into one arm of the U-tube, into which is then

\*FIG. 54.—Novy jar of large size for Petri dishes and numerous test-tube cultures. Clamped as when in use. Between the clamped parts is a rubber gasket, carefully waxed and vaselined. Darwin's wax-mixture is advised. The writer also usually wires in the waxed top parts. The gas inflow is cut off by twisting the uppermost (horizontal) ground-glass stopper, which must be carefully vaselined. One-third actual size.

crowded a tight-fitting, soft, rubber stopper. This end is finally buried for an inch or so in a small beaker of glycerin and is perfectly air-tight. A rimless test-tube about 5 inches (13 cm.) long and of a diameter such that it will just slip easily up the other arm of the U-tube, is now packed by means of a pencil or glass rod with 8 or

10 grams of pyrogallol acid, covered quickly with 25 cc. of 10 or 15 per cent caustic-potash water, and slipped up the open end of the tube, which is immediately plunged into a dish of mercury and held there (under a shelf) until enough of the oxygen is absorbed so that it will stay down of its own weight. The exposure should be made at 25° or 30° C., or at least at temperatures considerably above zero, since the absorption of the oxygen is slow in cool air.

The tube containing the pyrogallol acid and potash mixture floats on the mercury and rises, of course, in the arm of the U-tube as the oxygen is absorbed and the mercury enters it. This tube must not, therefore, be too long so as to hit against the curves of the U-tube before all of the oxygen has been absorbed; otherwise the mercury will pass up between the two tubes and overflow into the mixture. In other words, several centimeters must be allowed for the rise of the mercury.

A few experiments will determine how much of the mixture is necessary for a tube of a given bore and how long it takes to absorb all of the oxygen.† The level of the mercury in the open end with all the oxygen absorbed may be recorded by a scratch on the tube as a rough guide in subsequent work. At least half a dozen of these tubes will be found useful. They may be made in any laboratory or may be procured from dealers in glassware.

In the use of carbon dioxide, especially with sensitive organisms, two factors must be considered, (1) the simple exclusion of air, as in case of hydrogen, and (2) the change in the reaction of the medium due to absorption of the gas (formation of carbonic acid).

\*FIG. 55.—A simple device for growing organisms in air deprived of its oxygen. In the left arm are the cultures; in the right arm is a test-tube containing a mixture of pyrogallol and caustic-potash water. The beaker contains mercury. About one-third actual size. A modification of Ganong's apparatus for study of germinating seeds.

† Macé states that 1 gram of the pyrogallol and 10 cc. of the 10 per cent potash-water are sufficient for each 100 cc. of air space.

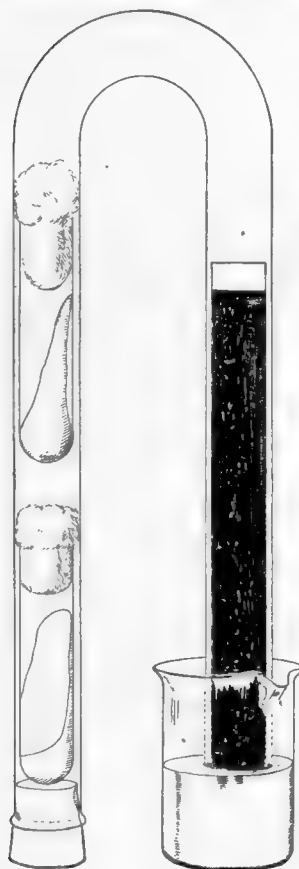


Fig. 55.\*

## LUMINOSITY.

Numerous saprophytic bacteria are luminous under certain special conditions. Luminosity is also a striking characteristic of at least one bacterial animal disease—the white disease, or sluggish disease, of sand fleas (*Talorchestia longicornis* and *T. megalophthalmia*), common on the shores of France and of Massachusetts at Woods Hole. Decaying potatoes and other vegetables are sometimes luminous. The question of luminosity should therefore be kept in mind by the student of plant diseases, although no luminous species are known to live in plants. Most of these interesting luminous bacteria have been found in salt water or near it, or on the flesh of quadrupeds and fish. Gorham has been able to grow them on strictly synthetic media. The most recent treatise is by Hans Molisch (*Leuchtende Pflanzen*, Jena, Gustav Fischer, 1904, pp. ix, 169, with 2 plates and 16 text figures).

Molisch records 26 species of luminous bacteria. He found that salt-water fish and the flesh of cattle exposed in the markets were very often luminous—48 per cent of 70 samples of the latter and nearly all the former. Of horse flesh 65 per cent and of cattle flesh 89 per cent became luminous on putting it into 3 per cent solution of sodium chloride, allowing a part of it to project into the air. Fresh-water fish are very seldom luminous. Seedlings exposed to Petri-dish poured plates curved heliotropically toward the light, but they did not become green. Other chlorides than that of sodium stimulate growth and light-production, *e. g.*, potassium, magnesium, or calcium chloride. Certain non-chlorides, such as potassium iodide, potassium sulfate, and magnesium sulfate have the same action (3 per cent or less). Potassium nitrate was also active on *B. phosphoreum* but not on *B. photogenus*. Manganese sulfate stimulated growth very noticeably but had no corresponding effect on the luminosity, which was weak. The spectrum of *B. phosphoreum* differs from that of the West Indian beetle, *Pyrophorus noctilucus*, and from that of a luminous fungus known as mycelium X. No biological importance is attributed to the luminosity which is ascribed to an hypothetical *photogen*. It is an oxidization phenomenon which can take place only in the presence of free oxygen. A temperature of 30° C. for forty-eight hours is sufficient to kill *B. phosphoreum* in gelatin cultures. The minimum temperature for this organism is below zero, the optimum is about 16° to 18° C., and the maximum is 28° C. The bacteria are luminous from minus 5° to plus 28° C. Light production is most intense from 5° to 20° C.

## FERMENTATION PRODUCTS.

The old conception of fermentation involves an evolution of gas (*fervere*, to boil), but the term is now used with a wider meaning. Like many other terms, it is difficult to use it always logically. In general, it means the breaking up of carbon compounds into simpler substances, either by the direct action of the protoplasm of the organism (hypothetical) or by chemical substances (enzymes, diastases) secreted by the protoplasm. Acids and alcohols are produced; gases may or may not be evolved. Other volatile products are also produced, *e. g.*, esters, but usually only in very small quantities. Certain of the bacterial fermentations are of large commercial importance, *e. g.*, the acetic, the lactic. The breaking up of albumen and

other complex nitrogen compounds, *i. e.*, putrefaction, is also sometimes called fermentation, and at present there is really no very sharp line to be drawn. Consult Green and Duclaux for the English and French views (Bibliog., XX). The student should observe :

- (1) Gases. Amount, rate of development, kinds (carbon dioxide, oxygen, hydrogen, nitrogen, marsh gas).
- (2) Acids. Volatile and non-volatile (lactic, acetic, butyric, etc.).
- (3) Alcohols (ethyl, methyl, butyl, glycerin, mannit, etc.).
- (4) Ethers and esters.
- (5) Aldehyds, sugars, gums.
- (6) Albumoses, peptones, amido-bodies.

The isolation and determination of the amount of these various products belongs to the province of the chemist, but the work should be done in the bacteriological laboratory and under the eye of the biologist if all sorts of errors, due to the unsuspected multiplication of intruding organisms, are not to creep in and render the work worthless. Only some crude determinations, as of proportion of the various gases evolved, may be made by the bacteriologist who is not a chemist. The volume of gas evolved from day to day may be measured in fermentation-tubes (fig. 49). Frost has devised a convenient gasometer for roughly estimating it (see his Laboratory Guide, plate I). These may be made in any laboratory out of cardboard.

If the gas is carbon dioxide it may be absorbed by shaking with 10 per cent NaOH. To do this, fill the bowl (fig. 49) even full of the strong caustic-soda water, place the thumb or forefinger over the mouth so as not to include any air, invert the tube so that the gas shall flow into the bowl and come into contact with the alkali, and shake vigorously until all of the carbon dioxide is absorbed. Tilt the fluid back into the open end, and remove the finger so as to equalize the pressure. If any gas remains after equalizing the air-pressure, place the finger over the mouth of the tube, tilt the gas into the bowl and apply a lighted match close to the mouth as the finger is removed. If it is hydrogen or marsh gas it will explode in the open end of the tube when the finger is removed and a flame applied. If it is nitrogen it will not support combustion (see Bibliog., XX, especially '90 Smith and '93 Smith).

How distinguish marsh gas from hydrogen?

Organisms easily inhibited by their own acid products may be kept alive a much longer time by adding a little calcium carbonate to the bouillon or agar.

In simple tests for acids, discard bright blue litmus paper, which is very sensitive to carbonic acid (try carbonated water on it), and use instead a good grade of reddish-violet (neutral) litmus paper. Such paper may be made in the laboratory (the best way) or may be purchased of H. Struers, Copenhagen.

#### ALKALIES (AMMONIA, AMINS, CARBONATES OF THE ALKALI METALS).

Determine rapidity of formation. Note that they are often masked by the simultaneous formation of acids. Try the litmus test and Nessler's test. Do not put Nessler's solution into the culture fluid, but expose it to steam from the culture. Observe the behavior of the organism when grown in peptone rosolic-acid water

with just enough HCl added to counteract the alkali in the peptone, and in neutral or slightly acid peptone-water or sugar-free bouillon containing acid fuchsin. On titration of acids and alkalies see Sutton (Bibliography of General Literature, IV).

#### REDUCING POWERS.

Determine rapidity of reduction of litmus, methylene blue, and indigo carmine in various fluids and solids (with and without grape-sugar). Probably all bacteria can reduce litmus, etc., but as the rapidity of reduction varies greatly in different species and in different media, it is desirable to make comparative tests. Consult a recent paper by Albert Maassen ("Ueber das Reduktionsvermögen der Bakterien, und ueber reduzierende Stoffe in pflanzlichen und tierischen Zellen," Arb. a. d. Kais. Gesundheitsamte, Bd. XXI, 3 Heft, 1904, pp. 377-384).

#### HYDROGEN SULPHIDE.

This gas is the product of a reduction. From what media and under what conditions is hydrogen sulphide given off with browning of lead acetate paper? This paper is readily prepared by dipping strips of white filter paper into a strong solution of lead acetate in distilled water. It should be kept in a tight tin box or a glass-stoppered bottle. Probably most, if not all, bacteria are able to produce hydrogen sulphide in nutrient media containing readily decomposable sulphur compounds. Is an enzyme necessary? When an organism grays potato cylinders in test-tubes, why is no hydrogen sulphide given off? The student should read papers by Petri and Maassen (Bibliog., XXVIII).

#### MERCAPTAN AND OTHER ODORS.

We need an odor chart to go along with our color charts. If we could have a set of standard substances with peculiar smells for comparison with the many odors evolved from bacterial cultures it would certainly be a great convenience. The difficulty at present is that the judgment of people varies greatly, in many instances, as to what the smell should be likened. As it is, the bacteriologist must do the best he can to define these penetrating smells, which are sometimes very characteristic of particular organisms. Some of the fishy odors are due to amines. Mercaptan is a very vile-smelling sulphur compound.

#### INDOL, PHENOL, LEUCIN, TYROSIN, ETC.

The production of indol is best studied in peptonized beef-broth naturally free from sugar or which has been deprived of its muscle sugar by growing in it (for a few hours only) *Bacillus coli* (Theobald Smith), after which it should be filtered clear. If *B. coli* or *B. cloacae* will not produce gas in beef-broth in the closed end of fermentation-tubes, it is free from sugar and suitable for this use. Many organisms give the indol reaction in Uschinsky's solution to which peptone has been added. The writer has never been able to obtain the indol reaction in any culture medium which did not contain peptone (using this word in the commercial sense.) Cultures which do not show the red reaction with sodium nitrite (0.02 per cent solution) and sulphuric acid at room temperature will frequently do so when put into hot water



for five minutes (70° to 80° C.). The browning of media due to excess of sodium nitrite must not be mistaken for this pink or red reaction. Uninoculated tubes should be included in the test, which may be made on the second and tenth day.

For methods of determining phenol see Lewandowski in *Deutsche Med. Wochenschrift*, 1890, p. 1186, and Chester's Manual, p. 33. Schmidt (Bd. II, p. 1008) gives the following as a qualitative reaction for tyrosin: Dissolve by boiling in water and add a solution of mercuric nitrate. The red reaction is sharper if a little fuming nitric acid diluted in water is added. Try also the violet reaction with neutral iron chloride.\* Leucin crystallizes in white soft scales.

REDUCTION OF NITRATES (AND MORE COMPLEX NITROGEN COMPOUNDS) TO  
NITRITES, TO AMMONIA, AND TO FREE NITROGEN.

For the pathologist the iodine-starch reaction is the most satisfactory test for nitrites, because it is not superlatively sensitive and consequently does not indicate

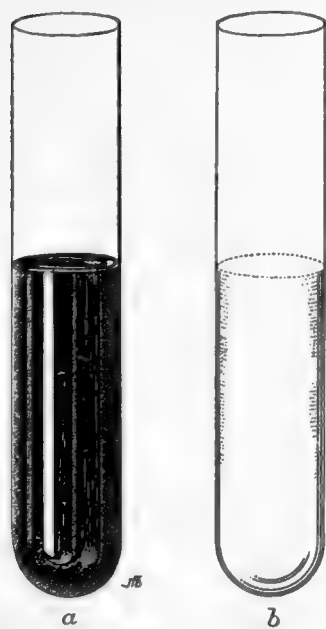


Fig. 56.†

traces of nitrite absorbed from the air. It is made as follows: Twenty-five cubic centimeters of distilled water are added to one-half gram (more or less) of pure potato starch and the fluid boiled. One cubic centimeter or more of this starch-water and 1 cc. of *freshly prepared* potassium-iodide water (1 : 250) are now put into the culture fluid, to which is then added a few drops of strong sulphuric-acid water (2 : 1). If any appreciable quantity of nitrite is present the culture immediately becomes blue-black from the liberation of free iodine, which acts upon the starch. Old potassium iodide water should never be used without first testing carefully, as it usually contains some *free iodine*. It is always best to first make a trial test without the bacteria. Commercial starch frequently contains products of bacterial decomposition and starch prepared aseptically should be substituted.

At least one-third of the organisms which have fallen under the writer's observation in recent years give the nitrite reaction when grown in peptonized

beef-bouillon containing potassium nitrate.

\*Mann (p. 323) gives the following as a specific tyrosin reaction: Denigès has recommended the well-known phenol aldehyde reaction for the detection of tyrosin. Nasse, in repeating Denigès' observations, has found the following to be a very delicate test for tyrosin, as neither proteids nor peptones give the color-reaction. Proceed thus: Add a few drops of formol solution to concentrated sulphuric acid, when, on warming with tyrosin, a brown-red color is obtained, which, on addition of acetic acid, becomes green.

†Fig. 56.—*Bacterium syringae* (van Hall). Nitrate bouillon cultures 5 days old, to each of which has been added boiled starch water, potassium iodide water, and sulphuric acid. In tube *a* the potassium nitrate was reduced to the nitrite, and on addition of the reagents free iodine was liberated, and the starch blued. In the other no nitrite had formed, no iodine was liberated, and the starch remained colorless. For discrepancy see text.

Fig. 56 shows how differently quite similar-looking cultures may react when submitted to this test. Both of these organisms were received from van Hall under the name of *Pseudomonas syringæ*, *a* being van Hall's own isolation and *b* being supposedly a subculture from Beyerinck's isolation. Neither one would produce any blight in lilac shoots.

There is no simple way known to the writer of distinguishing ammonia from the amins, as both react to Nessler's reagent. Nitrogen may be distinguished from the other gases of fermentation by the fact that it is not absorbed by sodium or potassium hydroxide and will not burn or support combustion. This gas is produced readily from nitrates by a number of green-fluorescent organisms (dung-destroyers) but not by all of them.

#### FIXATION OF FREE NITROGEN AND THE OXIDATION OF AMMONIA AND AMMONIUM SALTS TO NITRITES AND NITRATES.

These processes are probably common enough to organisms of the soil, many of which have not been investigated, but they are not known to be brought about by plant parasites exclusive of the root-tubercle bacilli of the Leguminosae, which some believe to be parasites (see Peirce).<sup>\*</sup> They are believed to be of rare occurrence in bacteria which grow well on ordinary culture media.

The nodules on roots of plants will hereafter be considered more fully. The reader should consult a paper by Geo. T. Moore on "Soil Inoculation for Legumes," Bureau of Plant Industry, United States Department of Agriculture, Bull. 71, January 23, 1905; and one by Maria Dawson, "Further Observations on the Nature and Functions of the Nodules of Leguminous Plants," Philosophical Transactions Royal Society of London, Series B, Vol. CXCI, pp. 51-67, 1900, with 2 plates.

#### ASSIMILATION OF CARBON DIOXIDE.

Some soil organisms are believed to obtain their carbon directly from carbon dioxide, and would thus be exceptions to the law that all non-chlorophyllous plants must obtain their carbon from organic substances. This supposition, while probably true, has not, we believe, been established satisfactorily. Its elucidation offers a most interesting line of research (see Bibliog., XXVI.)

#### PIGMENTS.

Bacterial growths are often bright colored, and an examination of the pigments should form part of one's study of an organism. They may be considered as follows:

(1) Under what conditions formed? Can they be eliminated by growing the organisms in the dark or under unfavorable conditions, *e. g.*, near the maximum or minimum temperature? *Bacillus prodigiosus* is a favorable organism for experiment.

(2) In what soluble (water, hydrogen-peroxide in water, ethyl alcohol, methyl alcohol, glycerin, acetic ether, petroleum ether, sulphuric ether, acetone, chloroform, turpentine, benzine, benzole, xylol, toluol, carbon bisulphide, etc.)? The pigment should be tested in as many solvents as possible.

<sup>\*</sup>Peirce, George James. The Root-tubercles of Bur Clover (*Medicago denticulata* Willd.) and of Some Other Leguminous Plants. Proc. Calif. Acad. Sci., 3d series, Botany, Vol. II, No. 10, San Francisco, Cal., June 21, 1902, pp. 295-328, with 1 plate.

(3) How are they acted on by acids, alkalies, and other reagents?

(4) Of what use are they to the organism? Are they oxidation-products? Examine spectroscopically, if possible.

On the addition of acids or alkalies, a bacterial pigment may remain unchanged, may be changed into some different color, may be destroyed, or may be converted into some colorless compound which will regain its original color on changing back the reaction. The yellow pigment of several species of *Bacterium* (*Pseudomonas*) remains unchanged in the presence of acids and alkalies. The blood-red color of *Bacillus prodigiosus* becomes carmine in the presence of certain acids and yellowish-brown in the presence of certain alkalies. The blue color of *Bacterium syncyaneum* is said to be produced only in acid milk. The beautiful green fluorescence of *Bacterium pericarditidis* (*Bacillus pyocyaneus pericarditidis*), and probably of all this group of bacteria, is produced only in alkaline media. According to Jordan two pigments are normally produced by many green-fluorescent organisms. The blue pigment pyocyanin is visible by gaslight and is soluble in chloroform. The green-fluorescent pigment is insoluble in chloroform and yellowish by gaslight. By this latter test the two can be distinguished when mixed. Soluble phosphates and sulphates are necessary for the production of green fluorescence. The ability to produce pyocyanin is easily lost. Its production in the culture-medium, unlike that of the fluorescine, is not dependent on the presence of phosphates or sulfates. Pyocyanin turns red with acids, fluorescine becomes colorless; both return to their original color on adding alkali sufficient to change the reaction. "Asparagin, ammonium succinate, ammonium lactate, and ammonium citrate all proved suitable for the development of the fluorescent pigment." The yellow and black pigments are the result of oxidations. (See papers by Gessard, Thumm, and Jordan, Bibliog., XXIII).

The pigments of bacteria range from one end of the spectrum to the other. Thus we have various shades of black, brown, violet, indigo, blue, green, yellow, orange, and red. Many bacteria produce no pigment, *i. e.*, are white when seen in mass. Others produce several distinct pigments. Many of the plant parasites are yellow, *e. g.*, *Bacterium campestre*, *Bact. phaseoli*, *Bact. hyacinthi*, *Bact. Stewarti*, *Bact. juglandis*. Some of these yellow organisms stain the host-plant and certain nutrient substrata a deep brown. Other plant parasites are white but also stain the host and certain substrata brown, *e. g.*, *Bacterium solanacearum*, *Bacillus carotovorus*, *B. aroideae*. Others are pure white and are apparently destitute of any pigment-producing powers, *e. g.*, *Bacillus amylovorus*, *B. tracheiphilus*. Very many bacteria when grown on cooked potato produce a gray stain in this substratum, especially in that part freely exposed to the air, *i. e.*, out of the water.

Some other color changes in the host should be mentioned. Various brown and red stains visible in certain plants when attacked by bacteria are not attributable directly to the presence of the microorganisms in the tissues. These are oxidation phenomena likely to occur when the plants are wounded or destroyed by any agent whatsoever. A few illustrations will make my meaning clear. When the limbs of pear trees are destroyed by blight the foliage becomes black, but this blackening

also occurs frequently when the flowers, green fruits, or foliage are killed by other causes. In the leaves of *Amaryllis atamasco* the writer obtained red stripes by injecting the yellow *Bacterium hyacinthi*, but no bacterial disease followed, and the same plant reddens when bruised. Broomcorn shows conspicuous red blotches when attacked by the broomcorn organism, but the parasite itself does not produce a red pigment, while the plant reddens easily as the result of aphid-punctures or wounds of any sort. Sugar-cane attacked by *Bacterium vascularum* shows a conspicuous red stain in the bundles, but other causes, such as the gnawings of an insect or the presence of a fungus, may lead to a similar stain, while the bacterium itself does not produce any red pigment.

#### CRYSTALS.

Determine the nature of the crystals observed in the various media. Many of these are double ammonium salts; others result from the action of trypsin on proteids. Crystals which are not due to the drying out of the media are common phenomena in old cultures of many sorts, especially if the media were not originally saturated with alkali (soda or potash). Fig. 57 shows two types of crystals formed in +15 nutrient agar by two green-fluorescent organisms received from van Hall as *Pseudomonas syringæ*, and a third type produced by the olive tubercle organism.



Fig. 57.\*

#### QUESTION OF EXISTENCE OF ENZYMES.

The enzymes of English writers are the diastases of Duclaux. They are chemical substances, the exact composition of which has not been determined. They may be regarded as the working tools of protoplasm. The following are some of the best known kinds:

- |   |                                      |
|---|--------------------------------------|
| (1.) Diastasic (starch-destroying).         | (5.) Lab or rennet (casein-forming). |
| (2.) Inverting (sugar-splitting).           | (6.) Lipase (fat-splitting).         |
| (3.) Cytohydrolytic (cellulose-dissolving). | (7.) Pectic (pectin-splitting).      |
| (4.) Proteolytic (peptonizing).             | (8.) Oxidases (oxidizing).           |

Trypsin is common. Pepsin is not known to be produced by bacteria and should be searched for.

Many bacteria invert cane-sugar, but invertase is believed to be rare. This, however, may be an ill-founded conclusion. The experiments of various animal physiologists have shown that when cane-sugar is injected into the blood-stream it is excreted unchanged, and according to Julius Sachs cane-sugar, inulin, etc., must

\*FIG. 57.—Crystals formed in cultures of *Bacterium syringæ* (van Hall). 1. From tube II, Aug. 14 (agar stock 693), from van Hall's II, *i. e.*, his own isolation corresponding to *a*, fig. 56. 2. From tube I, Aug. 14 (stock 693), from van Hall's I, which is from Beyerinck's old isolation (see *b*, fig. 56)  $\times 3$ . Nos. 1 and 2 drawn Aug. 30, 1902. 3. Crystals formed on slant litmus-lactose agar which was inoculated with the organism causing olive-knot. About one-half inch of slant in middle part of culture 1 month old, *i. e.*, made January 20, 1904; drawn February 17-19.  $\times 3$ . Temperature during growth, 20° to 25° C.

first be reduced to glucose (grape-sugar), before they can be used as food by plants. When no invertase has been detected the general hypothesis has been that this inversion was due to the direct action of the protoplasm, but the recent isolation by Buchner and others of an invertase (Zymase) from yeast, in which it was long believed that none existed, once more emphasizes the uncertainty of negative conclusions.

Diastase is common. Is there more than one kind, *i. e.*, a sort which can only convert the starch into amyloextrin and another which converts it into maltose and dextrine? In many cases, when the organism is grown on potato, the conversion is carried only a little way and stops, there being always a copious purple or red-purple reaction with iodine. In other cases, *e. g.*, when *Bacterium campestre* is grown on potato, the starch conversion is so complete that after a few weeks there is little or no color reaction when the potato-cylinder is mashed up and iodine water added. What makes this difference?



Fig. 58.\*

A substance capable of dissolving the middle lamella appears to be common to all bacterial plant parasites and a true cytase presumably occurs, but much additional study is necessary. Probably several enzymes are confused under this name, just as several chemically different substances are still called "cellulose." The substance which dissolves the middle lamella in some cases is probably ammonium oxalate. The writer has not been able to dissolve it by means of pure oxalic acid, but that of turnips softens in ammonium oxalate.

The lab or rennet ferment is rather common. Its action should not be confused with the curdling of milk due to the formation of acids. Tests may be made in litmus milk. Is there more than one kind of such ferment? Some organisms coagulate the milk promptly into a solid mass which finally shrinks, extruding whey. Others cause the casein to separate out of the fluid very slowly as a

multitude of separate particles which only become compacted very slowly.

The writer has not met with the oxidizing enzymes, unless the substance in bacterial cultures which causes rapid evolution of oxygen from hydrogen peroxide is such an enzyme, as Dr. Loew maintains (Bibliog., XLV). Many other enzymes undoubtedly occur and play their part. The student should search for emulsin, lipase, lactase, maltase (glucose), etc.

All known enzymes when freely exposed to steam heat are destroyed at temperatures considerably under 100° C. They are less sensitive to heat than the bacteria themselves, but are destroyed by a few minutes exposure to temperatures 15° to 30° C. (moist heat) above the thermal death-point of the organisms which have produced

\*Fig. 58.—Thick-walled Kitasato flask for filtration or evaporation *in vacuo*, etc. Much reduced.

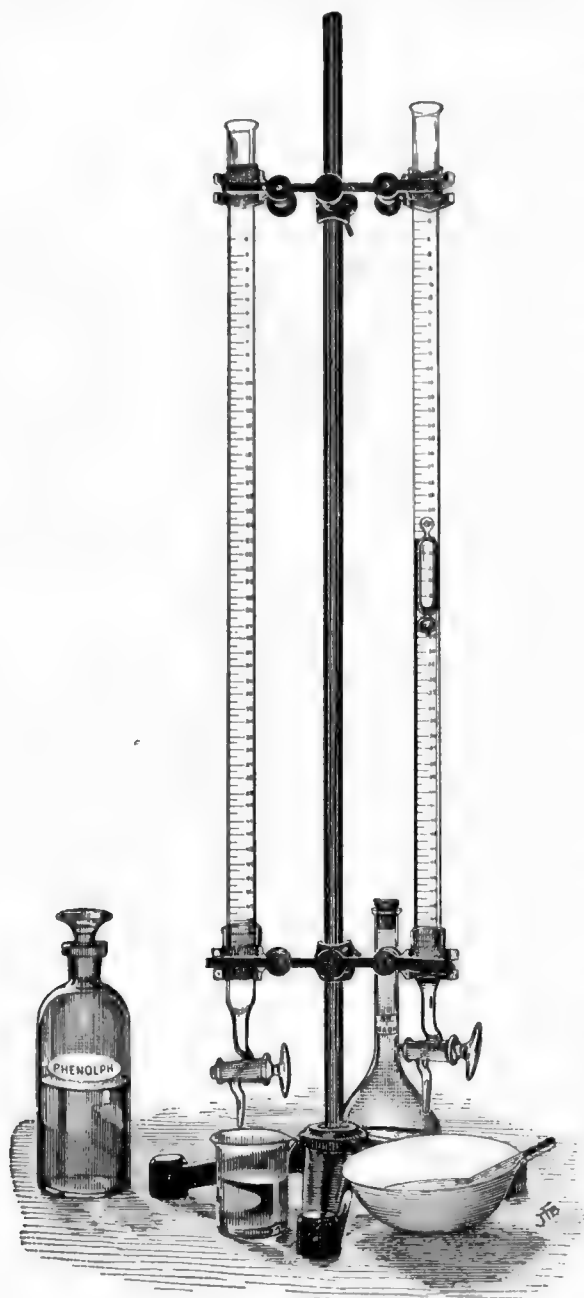


Fig. 59.†

\*The same amount of dry heat does not affect them, and Loeffler has recently advised exposure of thoroughly air-dried tissues and cultures to 150° C., dry heat, as an easy way of eliminating the bacteria prior to grinding and extraction of the uninjured enzymes and other soluble products. Non-sporiferous bacteria may be heated at 120° C. for 2 to 3 hours. Tissues and sporiferous bacteria should be heated at 150° C. for one-half hour. (Deutsche Med. Wochenschrift, Dec. 22, 1904.)

†FIG. 59.—Burettes used by the writer for titrating culture media. Twentieth-normal sodium hydrate is used to determine the acidity, and the medium is finally brought to the desired alkalinity with quadruple-normal sodium hydrate. The fluid is boiled and titrated hot, using phenolphthalein as the indicator. The burettes should be graduated to tenths of a cubic centimeter and should hold 50 cc. Alkali should not be allowed to stand in them.

them.\* Some of them are very sensitive to the presence of acids, alkalis, strong alcohol, or antiseptics, or their action is inhibited by the presence of other enzymes or of products of enzymic fermentation in excess, or by the absence of some combining substance, such as lime or some weak acid. Some do not pass readily through the Chamberland filter or through filter papers. Some are destroyed at lower temperatures after precipitation. Some are not produced except in presence of the substance which they can decompose, but this is not true of all. Usually an organism produces more than one ferment and some bacteria are known to produce five or six. *Bacterium campestre* produces at least three and probably four, viz, diastasic, cytohydrolytic, proteolytic, and rennet. It also inverts cane-sugar, but it is not yet known whether this change is accomplished by means of an invertase. On enzymes derived from bacterial soft-rot organisms the reader should consult recent papers by Jones (Centralb. f. Bakt., 2 Abt., and Vermont Exp. Sta. Rep.). Levy has published an interesting paper on "Some physical properties of enzymes" (The Jour. Infect. Diseases, Vol. II, 1905, pp. 1-48).

For concentrating fluids in vacuo at low temperatures (50° to 60° C.) the thick-walled Kitasato flask shown

in fig. 58 is very convenient. The side tube is attached to the suction-pipe of an air-pump and into the neck is thrust a rubber stopper carrying a thermometer and a U-shaped glass tube of small bore, the outer arm (36 inches long) ending in a beaker of mercury. Heat may be applied by means of a water-bath. By substituting a funnel for the thermometer the same device may be used to hasten the filtration of thick liquids, hard-pointed filter papers being employed.

#### SENSITIVENESS TO PLANT ACIDS.

The tests should be made with malic, citric, lactic, oxalic, and tartaric acids added to neutral beef-broth, peptone-water, or plant-broths, or to synthetic media (see Am. Nat., 1899, p. 208). It is best to titrate with  $\frac{N}{10}$  or  $\frac{N}{20}$  solutions, to acidify with  $\frac{2N}{1}$  or  $\frac{4N}{1}$  solutions, and to reckon the acidity in cubic centimeters of normal

solution ( $\frac{N}{1}$ ) required per liter of medium. If preferred, it may be calculated on 100 cc. portions and expressed in per cents, but there is no advantage in this, and it has the disadvantage of introducing fractions.

#### SENSITIVENESS TO ALKALIES (POTASSIUM OR SODIUM HYDRATE).

Determine in each case the optimum reaction of the medium for growth. For the majority of bacteria this is said to lie between +10 and +15 of Fuller's scale.† The best neutral litmus paper should be used freely, but acid and alkaline media should be titrated with phenolphthalein and  $\frac{N}{10}$  or  $\frac{N}{20}$  solutions. In some media—*e. g.*, gelatin, juices of various plants—the end-reaction with phenolphthalein and caustic soda is not very sharp. In these cases the titration should be stopped at the first trace of change of color. If one adds alkali until the fluid is decidedly red, then a distinct statement to that effect should be made, since otherwise no comparisons of any value can be made. All of the writer's + and – references to media are based on a reaction stopped at the first distinct trace of pink color. As much again alkali must sometimes be added to obtain a deep-red color.



Fig. 60.\*

\*FIG. 60.—Stock bottle of  $\frac{N}{20}$  sodium hydrate solution. The small bottle at the right holds concentrated potash liquor to remove the carbon dioxide from the air which enters the bottle. About one-fourth actual size.

†The plus and minus on Fuller's scale denotes, respectively, acid and alkaline media. The +10, for example, means that exactly 10 cubic centimeters of normal alkali must be added to a liter of the culture medium to render it exactly neutral to phenolphthalein, and, correspondingly, –10 means that the fluid is alkaline to phenolphthalein and that 10 cc. of normal acid would need to be added to bring 1 liter back to the neutral point. The student should not confuse the litmus neutral point and the phenolphthalein neutral point, as they are about 23° apart, *e. g.*, +10 of Fuller's scale (acid side) is distinctly alkaline to litmus. (Consult '95, Fuller, Bibliog., XVI.)



The writer has used the foregoing method of determining the reaction of culture media for several years and has, in general, found it exceedingly exact and valuable, but it does not appear to be well adapted for determining the amount of alkali (ammonia and amines) produced by bacteria in culture media (see Sutton, Bibliog., IV). The apparatus required to make these titrations is shown in figs. 59 and 60.

Some experiments recently made by the writer with *Bacillus tracheiphilus* in peptonized beef-bouillons of varying degrees of acidity (acid of beef-juice) and alkalinity seem to show that toleration of sodium hydrate can be considerably increased by inoculating each time from alkaline bouillons rather than from acid ones. Taken from +20 bouillon (descended from +20 bouillon) this organism would cloud the same bouillon only down to 0; taken from 0 or -5 bouillons (descended from -2.7 bouillon) it would cloud the same bouillon down to -10 and probably farther, but not to -20. Bouillon containing various amounts of c. p. sodium chloride behaved in the same way. The organism would tolerate the largest amount of salt (1.5 to 2 per cent) when first grown in an alkaline bouillon. When inoculated from a +20 bouillon the organism finally grew in 1 per cent salt bouillon, but only after a decided retardation, and would not grow at all in +15 peptonized beef-bouillon containing 1.5 per cent sodium chloride.

Bacteria vary greatly in their toleration of acids and alkalies, the range of growth being from *minus* 100 (or more) of Fuller's scale to *plus* 100 (or more). The limits of growth are not known, but it is probable that the extremes of toleration in particular aberrant species is much greater than that here given, *e. g.*, on the acid side in sulphuric acid and vinegar bacteria, and on the alkaline side in case of those organisms which are able to grow in the lime-vats of tanning establishments and in alkaline springs. Lehmann & Neumann ('96, Bibliog., III), state that they have found bacteria that will endure 100 cc. of normal acid per liter of fluid culture media, *i. e.*, equal to about 1 per cent sulphuric acid. Some species are indifferent to a considerable degree, having a wide range of growth either side of the (phenolphthalein) neutral line; others prefer alkaline media; others acid media. Many are extremely sensitive to their own acid products (acetic, lactic, butyric, etc., acids). Not a few are differently affected by different acids and alkalies. Every new organism presents a whole series of special problems.

#### EFFECT OF DESICCATION.

Drops of fluid cultures or small masses of gelatin or agar cultures are spread on small ( $\frac{1}{4}$ -inch) clean, sterile cover-glasses, in covered sterile Petri dishes, and are set away in the dark, in dry air (a dry room). The test is finally made by seizing one of these covers with a pair of sterile forceps and dropping it into a tube of sterile bouillon or other medium of a stock previously determined to be exactly adapted to the growth of the organism, *i. e.*, one which does not exert upon it any retarding influence. Occasionally a tube will become contaminated, but enough must be inoculated so that this will not affect the final result (20 at one time is not too many). Fluid cultures are preferred. Solid cultures do not give strictly comparable results.



Organisms believed to be non-sporiferous show great differences, some being killed by an exposure of a few minutes or a few hours, while others remain alive for many weeks. For further information see the special chapters on *Bacillus tracheiphilus*, *B. carotovorus*, *Bact. hyacinthi*, etc. Tests may also be made in air dried over sulfuric acid or calcium chloride. Harding & Prucha have shown recently that *Bacterium campestre* remains alive much longer when dried on cabbage seed than when dried on glass cover-slips. In their experiments this organism was dead on glass at the end of ten days, but alive on seed at the end of thirteen months.

## EFFECT OF DIRECT SUNLIGHT.

The exposures should be made in a thin stratum of nutrient agar, not sowed too thickly (there may be several hundred colonies on the plate, if properly distributed), in thin-bottomed Petri dishes, to an unclouded sun for 5, 10, 15, 30, 45, and 60 minutes, a portion of the bottom of the plate, which is placed uppermost, being covered by some substance impervious to light, such as several folds of Manila paper



Fig. 61.\*

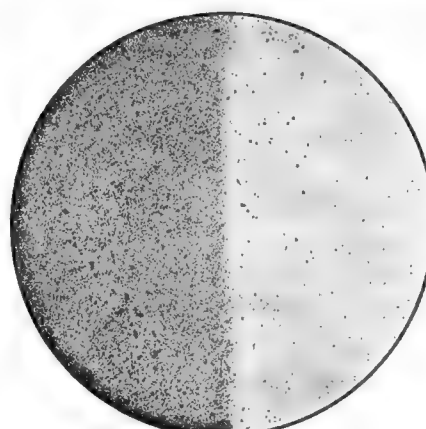


Fig. 62.†

or of the black paper which comes wrapped around photographic dry plates, covered in turn by white paper. Exposures of several hours are not recommended. If the layer of agar is very deep, or if the sowings are too thick, some organisms will screen others and all will not be killed. Ten cubic centimeters is a proper amount of agar to use for a plate having an area of 60 square centimeters. The latitude, altitude, time of year, time of day, and intensity of the light should also be recorded. In the summer-time it is very important that the exposures should be made on blocks of ice or,

\*FIG. 61.—Gelatin culture of *Bacillus amylovorus* (Burrill) Trev. in a Petri dish. Exposed in 1896 to direct sunlight for four hours on ice after covering portions of the plate with pasteboard figures. The bacteria grew only under the protected parts. Drawn from a photograph made after five days incubation of the culture at about 24° C. The temperature of the gelatin during exposure was about 25° C. Three-fifths natural size.

†FIG. 62.—Agar culture of *Bacterium phaseoli* (Erw. Sm.) in a Petri dish. Right one-half exposed to direct sunlight for thirty minutes, on ice, the other half protected by several folds of Manila paper. Dish then set away in the dark for several days. One-half natural size. The scattering colonies on the right side undoubtedly grew from bacteria which were sheltered from the direct rays of the sun by overlying organisms, i. e., the plate was sown too thickly.

better, on larger Petri dishes filled with pounded ice; otherwise, in case of 30 to 60 minute exposures, the temperature may rise nearly or quite to that of the thermal death-point of the organism, and then we shall have the effect of heat complicating that of light. To avoid errors it is always best to take one-half of each dish as a check (rather than the whole of a separate dish), and the rise of temperature should be carefully recorded. In some tests made by the writer in Washington in May the temperature of the plates exposed in the open air to the sun for 45 minutes (without ice) rose from 25° to 51° C. Figs. 61 and 62 show the effect of sunlight upon thin sowings of *Bacillus amylovorus* and *Bacterium phaseoli* in poured-plate (Petri-dish) cultures.

#### VITALITY ON VARIOUS MEDIA.

By this I mean the determination of the resistance of organisms to their own decomposition products. This varies greatly. Much may be learned by the study of old cultures. Do not discard test-tube cultures until after many weeks. Examine frequently. Make transfers from tubes which have been inoculated for a year or more. Determine whether this vitality is due to spores or persists in the ordinary vegetative rods. On what kinds of media does a particular organism live longest? Can length of life be increased by occasionally neutralizing decomposition products (acids) with sterile carbonate of lime? or by occasional additions of food? Some bacteria are veritable revelers in filth; others are extremely sensitive; all are soon under abnormal conditions in our culture-tubes.

Another way of keeping bacteria alive for a long time is by reducing their growth to a minimum. Stock-cultures, especially of perishable organisms, should, generally speaking, be kept in the ice-box at temperatures under 15° C. This greatly reduces the always heavy burden of keeping alive cultures of organisms which are not in immediate demand for actual experiment. Some will also remain alive a long time when sealed airtight. Particular organisms may be kept a long time in particular media, *e. g.*, *Bacterium vascularum* in diluted peptonized cane-juice gelatin, *Bact. Stewarti* in milk, etc. Some organisms are quite resistant to their own decomposition products, *e. g.*, *Bacillus coli*, *Bact. pericarditidis*. In the cool box *B. coli* will often live a year in agar stab cultures.

#### MIXED CULTURES AND MIXED INFECTIONS.

The behavior of mixed cultures and mixed infections may be tested in various fluids, making poured plates from time to time; in tubes of agar, potato, and other solid media; in crossed streaks on agar or gelatin plates; and in the plants themselves.

When two bacteria, or a bacterium and a fungus, are sown together in a culture-medium, there may be (1) antagonism, with the crowding out of one species; (2) a more or less complete indifference, both organisms growing well; or (3) a distinctly favorable effect, *i. e.*, a marked increase in growth or in pathogenic effect due to the presence of the second organism. The antagonism may result in the prompt destruction of one of the organisms, or only in a retardation or inhibition which finally disappears after the first organism has made its growth and subsided. In some cases the favorable effect of one organism upon another is due to the fact that it prepares food for it out of an unfavorable substratum, *e. g.*, maltose from starch.

In the plant one organism often paves the way for others which complete the destruction, *e. g.*, *Bacterium campestre* and *Bact. solanacearum* are often followed by soft white rots. Some of the latter, however, are able to make their way unaided, a fact observed and known to the writer for a white rot of the cabbage as long ago as 1896.

The simplest way of studying the antagonistic action of bacteria is by means of crossed streaks on agar or gelatin plates. These may be made either simultaneously, or one after the other has begun to develop. The action of the antagonistic organism may also be obtained by letting its products diffuse through a collodion sac into bouillon inoculated with the other organism. In practice, the bottom of a test-tube is removed and a collodion sac is securely fastened in its place. This tube is filled with the usual quantity of bouillon and lowered into a larger receptacle (tube or flask), the collodion part being surrounded by bouillon. The inner and outer receptacles are now plugged with absorbent cotton, and the apparatus is sterilized in the steamer or autoclave. The two tubes are then inoculated simultaneously, or the outer one some hours or days after the inner one. (See an interesting paper on Antagonism, by Frost, in *Jour. Infect. Diseases*, Vol. I, 1904, pp. 599-640). Frost has also devised two new methods for studying this subject, viz, the divided-plate method and the agar-block method. The first is a modification of the ordinary streak method. It is managed as follows: A Petri dish is divided into two equal parts by means of a glass rod fastened to the bottom with collodion. A tube of melted agar is inoculated with the antagonistic organism and poured into one half of this plate. Into the other half sterile agar is poured. Streaks of the other organism are now made crosswise of the hardened surface. If there is marked antagonism there will be a decided difference in the behavior on the two sides of the plate, *i. e.*, on the sterile agar as compared with the inoculated. To insure a uniform streak the inoculated loop should be swept across one half of the plate, then re-inoculated and swept across the other half of the plate.

The method by agar-blocks consists in substituting agar-walls for collodion walls. A sterile 3-cm.-deep Petri dish is poured full of nutrient agar. When it has solidified it is cut into rectangular blocks, 1 by 1 by 3 centimeters, using a sterile knife and taking all possible precautions to avoid contamination by air-borne organisms. A platinum needle is now dipped into a culture of the supposed antagonistic organism and thrust into the block lengthwise but not entirely through it. The mouth of the needle-track is sterilized and sealed by touching it for a moment with a red-hot iron. The head of a small wire nail set into a suitable handle will answer the purpose. The block is picked up with sterile forceps and dropped into a tube of sterile bouillon, which then may be inoculated with the other organism. More than one block and tube should be inoculated, and it is best to test the sterility of the outer surface of the agar-block by delaying the inoculation of the bouillon for a day or two after the inoculated agar-block has been dropped into place.

Still another method has been described by Frankland and Ward. They use the walls of a Chamberland filter to keep the bacteria separate. Bouillon for the one

organism is placed in a flask or large tube. That for the other organism is placed inside a Chamberland filter, which is then sunk into the other receptacle, whereupon it is sterilized and inoculated as in the collodion-sac method.

The favorable influence of a second organism may be studied in crossed streaks on sterile raw potato, carrot, turnip, etc.; on starch jelly; or on agar, gelatin, or silicate jelly with addition of varying amounts of the different plant acids, or plant juices, or other vegetable substances. Frost's divided Petri dish may be used for the jellies.

#### REACTION TO ANTISEPTICS AND GERMICIDES.

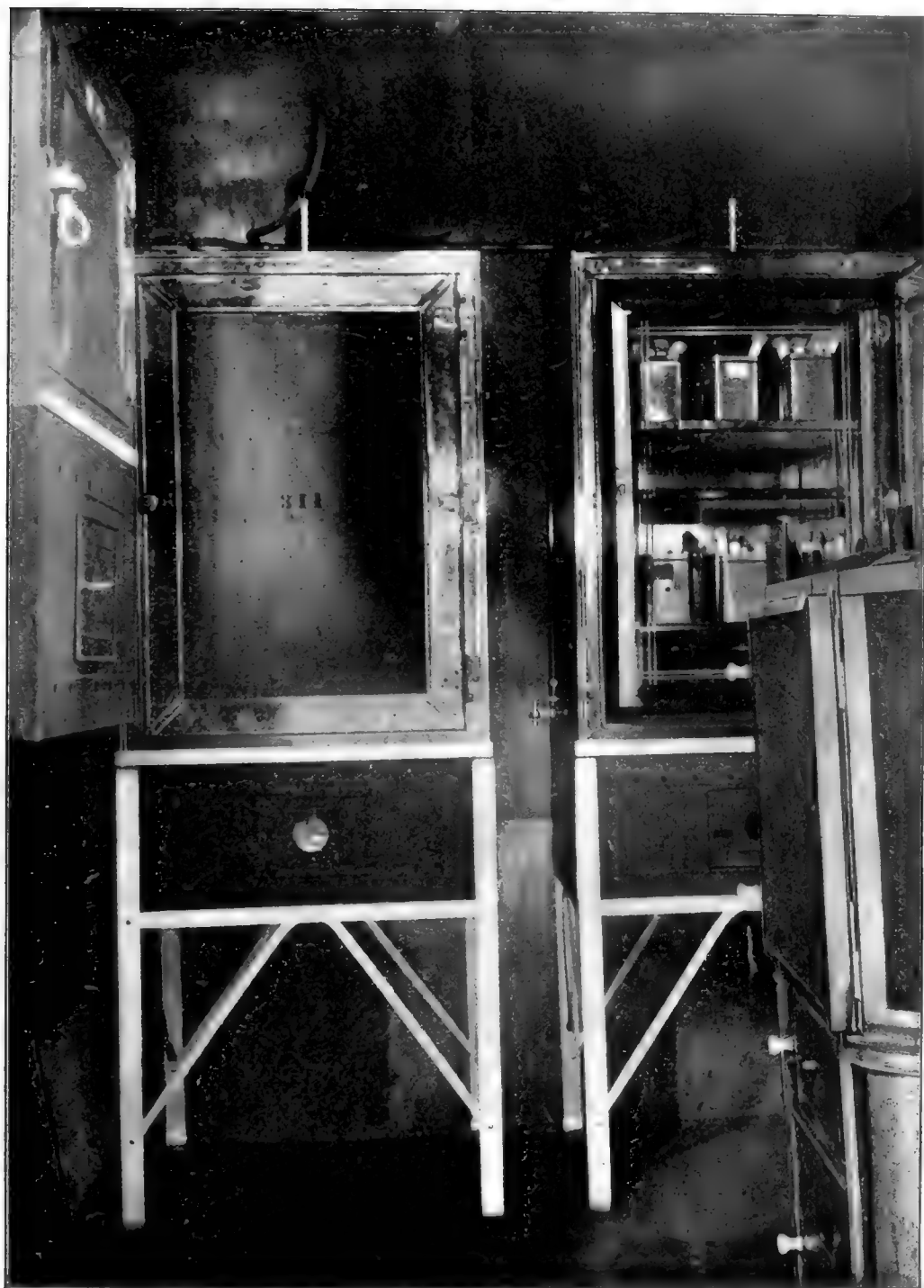
Antiseptic has been defined recently by Duclaux as follows: Any substance the intervention of which modifies in any form whatsoever the march of the phenomena (Bibliog., XX, Fermentation alcoolique, p. 461).

I still use the word with its old primary meaning (*anti*, against, and *sepsis*, decay). In this sense an antiseptic is any substance which prevents the multiplication of bacteria in putrescible substances. Large doses of antiseptics often exert a germicidal action, but such action does not necessarily follow. Often when the antiseptic substance is removed or diluted beyond a certain point growth takes place. The first seven substances mentioned below possess very active germicidal powers and are antiseptic in correspondingly small doses; the remainder are more or less valuable antiseptics, but are not valuable germicides.

- |                              |                                 |                        |
|------------------------------|---------------------------------|------------------------|
| (1.) Mercuric chloride.      | (5.) Lysol.                     | (9.) Benzoic acid.     |
| (2.) Sulphate of copper.     | (6.) Trikresol.                 | (10.) Salicylic acid.  |
| (3.) Formaldehyd (formalin). | (7.) Methyl violet (Pyoktanin). | (11.) Chloroform.      |
| (4.) Phenol (carbolic acid). | (8.) Thymol.                    | (12.) Sulphuric ether. |

This list may be extended indefinitely. The student should consult valuable digests in Sternberg's Text Book of Bacteriology and in Miquel & Cambier's *Traité de Bactériologie*. Some caution must be used in drawing conclusions from experiments. Mercuric chloride does not always destroy when the culture medium contains albuminoid substances. Sulphate of copper is more active in water than in bouillon.\* Some organisms will grow in a solution saturated with thymol (*e.g.*, in bouillon). Others will grow in the presence of chloroform (5 cc. of chloroform in test-tubes with 10 cc. of milk or beef-bouillon). Ten organisms have been found by the writer which, under the conditions named, grew in the presence of chloroform and two which grew vigorously in the presence of thymol. Russell reports one capable of growing in the presence of sulphuric ether. It is, therefore, not always safe to depend on these substances as antiseptics. Newcombe has made the same observation (Cellulose Enzymes, Annals of Botany, Vol. XIII, 1899, p. 60). In the opinion of the writer the statements of physiologists respecting the existence of enzymes in the tissues and fluids of the higher plants and animals must be taken with much allowance when chloroform, thymol, and similar antiseptics have been

\*Moore, George T., and Kellerman, Karl F. A Method of Destroying or Preventing the Growth of Algae and Certain Pathogenic Bacteria in Water Supplies. U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 64, 1904, pp. 44; see also Bull. 76, Bureau of Plant Industry. Certain pathogenic bacteria, such as *Vibrio cholerae* and *Bacillus typhosus*, are destroyed within a few hours in water containing traces of copper salts or dissolved particles of metallic copper.



A thermostat-room.

In the center of the building and lighted by electricity. Ventilated in the same way as the photographic dark-rooms, i. e., by an exhaust-fan run by an electric motor. Three of the thermostats were made by Bausch & Lomb, the fourth (felted) is a Rohrbeck.



depended upon to keep the solutions free from bacteria. This has been the case very frequently, and in several places in Greene's interesting book on Fermentations, published in 1899, it is said or inferred that the addition of chloroform will prevent the growth of bacteria. This might or might not be true; much would depend on the kind of organisms present. The medium to which chloroform or thymol has been added must be shut in and shaken continuously if the full antiseptic value of these substances is to be obtained.

#### THERMAL RELATIONS.

The student should determine—

- (1) Maximum temperature for growth (thermostat).
- (2) Minimum temperature for growth (ice-box).
- (3) Optimum temperature for growth (room or thermostat).
- (4) Thermal death-point (ten minutes exposure in the water-bath, in thin-walled test-tubes of resistant glass having a diameter of 16 to 17 mm., ordinarily in 10 cc. of moderately alkaline peptonized beef-bouillon, viz, +15 of Fuller's scale).
- (5) The effect of freezing (exposure to liquid air or to pounded ice mixed with coarse salt).

Thermal relations are among the most interesting and should be studied with great care in case of every organism. They offer valuable means of differentiation and also very useful suggestions as to geographical distribution and habitat. Good thermostats are made by various people. Several items of construction are important. The water or oil jacket should be of considerable volume (thickness) so as not to change temperature quickly; the cover should be thick and of the best non-conducting substances. The opening for the thermo-regulator should be at least  $1\frac{1}{2}$  inches in diameter (so as to take a Roux metal-bar thermo-regulator); the warm chamber should be of good size; the space beneath should be high enough between floors to accommodate any pattern of safety burner; and last, but not least, the workmanship should be of the very best quality, so that the apparatus will not leak. Nearly every worker has probably had experience with leaky thermostats at some time in his life and knows what a vexation of spirit they cause, particularly if filled with oil. A very excellent kind of thermostat is the old, large-pattern, felt-covered instrument devised by Dr. Hermann Rohrbeck and figured in the lower right-hand corner of plate 8. This plate shows a thermostat room with four thermostats in use. All are provided with Roux metal-bar thermo-regulators and Koch safety burners. One is for quick shifts as needed; and others are generally kept at  $30^{\circ}$ ,  $37\frac{1}{2}^{\circ}$ , and  $40^{\circ}$  or  $43^{\circ}$  C. These temperatures, in conjunction with the cool boxes, thermal baths, and various room temperatures, enable one to quickly determine the thermal relations of an organism. The height of the room is 10 feet, its depth 7 feet, and its breadth 5 feet 3 inches. A larger room would be more convenient. Such a room should be located and constructed so as to be as little subject as possible to external changes of temperature. It should be lined with asbestos and sheet iron, and *efficient* safety burners should be used to the exclusion of all others (see Lautenschläger's catalogue). The improved Koch safety burner is probably the best. All burners require frequent inspection.



The writer has no very satisfactory way of making exposures for determining the minimum temperature for growth. His method is to make such exposures in the bottom of a large, well-filled ice-box, which is opened as little as possible during the progress of the tests, and then only for the briefest periods. The degree of cold

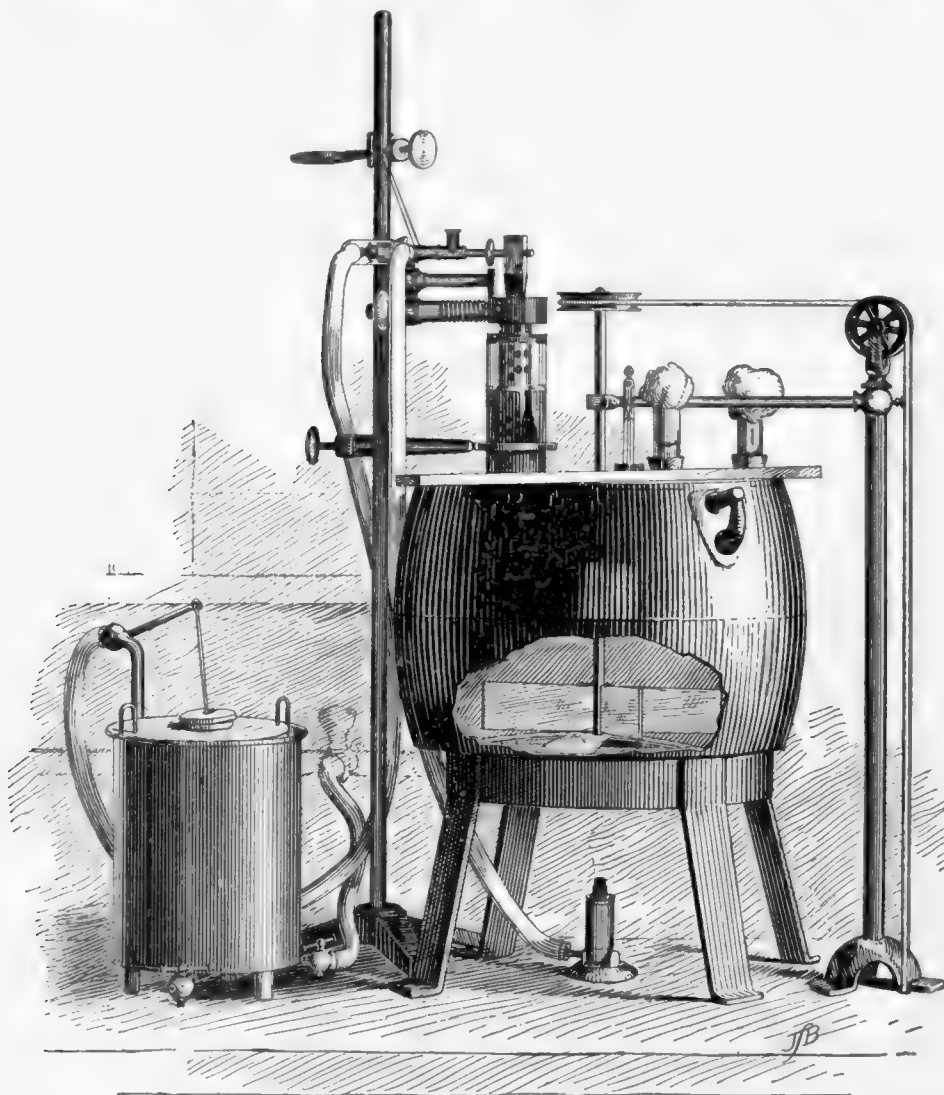


Fig. 63.\*

is governed by the amount of ice. A good thermometer is exposed in the midst of a bundle of inoculated tubes, and if the temperature shows any tendency to rise more ice is added. Under the most favorable circumstances the temperature of the

\*FIG. 63.—Modification of the Ostwald water-bath used by the writer for thermal death-point experiments. This consists of a porcelain-lined pot 11 inches in diameter at the top. This is filled with water kept in motion by a water-wheel turned by electricity. The heat is applied by means of a Friedburg burner and is controlled by Roux's thermo-regulator. Murrill's gas-pressure regulator is shown at the left.



air in the bottom of the chest may be kept fairly constant for some days or weeks, but with marked external fluctuations of temperature trustworthy results can be obtained only by constantly watching the box. What one needs for this work is a good-sized room kept at  $0^{\circ}\text{C}$ ., or a little below, in which thermostats may be installed at temperatures a little above freezing, *e. g.*,  $+2^{\circ}$ ,  $+5^{\circ}$ ,  $+7^{\circ}$ , etc. It would then be very easy to determine the minimum temperature at which any organism will grow—as easy as it is now to determine the maximum. Different levels in the same room may afford constant and useful differences in temperature.

The thermal death-point, which is a purely arbitrary standard, depending on the age and kind of culture, its volume, and the length of exposure, as well as the temperature, is when properly determined not least valuable. The writer, following that one of Dr. Sternberg's methods which is easiest to carry out, uses 10 cc. portions of moderately alkaline ( $+10$  or  $+15$ ) peptonized beef-broth† in test-tubes of uniform diameter (16 to 17 mm.), inoculates from recent bouillon-cultures with care not to touch the sides of the tube above the fluid, thrusts the tubes deep into the hot water, and exposes for ten minutes. All who make this test are urged to use standard alkaline beef-bouillon (for all organisms growing well in this medium) and to limit the exposure to exactly ten minutes, so that easy comparisons may be made. The five minutes exposure which has been recommended by some authors is rather too short, since it only a little more than suffices to warm the fluid up to the required temperature. Inoculation while the tubes are in the bath and after the fluid has been brought to the required temperature is inconvenient and has no special advantage.

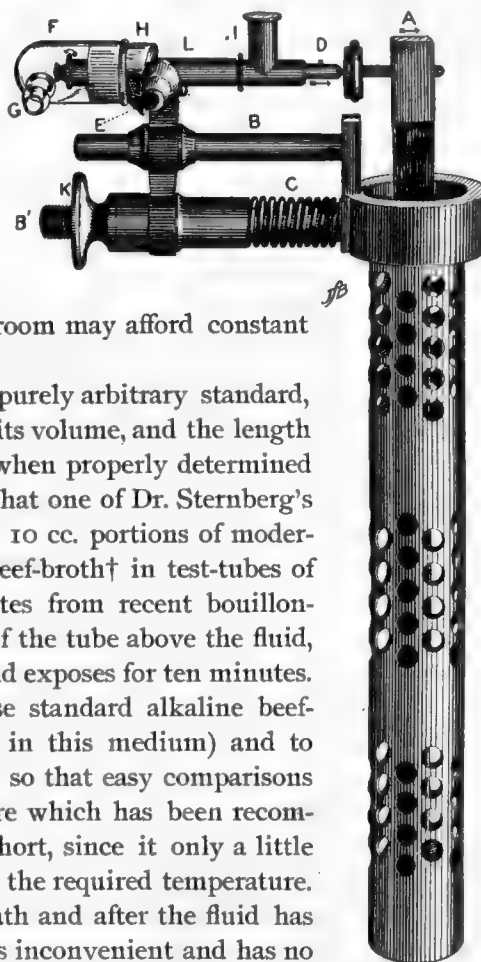


Fig. 64.\*

\*FIG. 64.—Roux's thermo-regulator, made by Maison Wiesnegg (P. Lequeux), Paris. The parts requiring description are as follows: A, bar composed of two metals (which expand and contract unequally) attached at bottom and free at the top, which moves with increased heat in the direction of the arrow; B, arm on which the upper part of the apparatus moves freely when K is turned; C, stiff spring; D, long rod which controls the gas-inflow, and the spring movement of which is in the direction of the arrow except when controlled by the counter movement of A, due to lessened heat; E, gas-inflow; F, gas-chamber, of glass; G, gas-outflow, to the burner; H, rubber stopper; I, cylinder screwing into L, and provided with capped upright tube filled with vaseline to prevent gas from escaping in the direction of D. The button shown in the gas-chamber at the left is part of D, and the gas enters the chamber between it and the left end of L, the size of the opening, and consequently the amount of gas, varying with the slightest movement of A. Different temperatures are obtained by turning the button K. The constant gas-flow is provided for by a small opening on the lower side of L, at its extreme left, in the gas-chamber. About two-fifths actual size.

†The thermal death-point in acid media is considerably higher—at least that of several organisms which have been tested in the author's laboratory.

An excellent water-bath is that known as the Ostwald-Pfeffer. The experimenter may, however, construct one for himself out of a medium-sized, thick-walled, porcelain-lined iron kettle (fig. 63). This should rest on a ring of heavy strap-iron supported by four stout iron legs. The burner required may be Dr. Friedburg's safety burner (a very inexpensive and good pattern). The thermo-regulator may be a common Reichert if the mercury seal is cleaned from oxide frequently. In such regulators a sharper contact and a longer freedom from obstruction is said to be obtained (Dr. Harris) by putting a drop of olive oil on top of the mercury. A much better instrument is the metal-bar mechanism known as the Roux regulator (fig. 64). This may be procured from the Maison Wiesnegg, in Paris. It should be kept from direct contact with the water and consequent rusting by burying it in a close-fitting glass tube filled with olive oil or glycerin. This tube is then sunk deep into the water and clamped to the wall of the kettle, which should have perpendicular sides. The water is kept in motion by means of a horizontal paddle-wheel at the bottom of the kettle. This consists of four light, oblique zinc or copper vanes (nickel copper is preferable) soldered to a long central rod which fits into a socket, below, and near its upper end passes through a hole or loop in a horizontal metal arm (a foot or less above the kettle), the other end of which is clamped to the upright rod of a solid iron tripod, or fastened to a rod bolted to the table. If compressed air can be had, a stiff cardboard windmill fastened to the upper end of the vertical rod completes the mechanism. The central part of the wind-wheel may be of cork. The vertical rod may be a piece of glass tubing, in which case it is cemented into a socket of the short metal post to which the vanes of the water-wheel are soldered. If a wind-wheel is attached, it is more convenient to have the vertical rod in two parts, fastened by a coupling. The rod, with its water-wheel attachment, may also be turned by some electrical device. The latter is the most convenient method. In fig. 63 the electric motor is not shown. This stands in a small box screwed to the under side of the table at the right. The switch is fastened to the wall above and back of the top of the thermo-regulator. The pulley band is of smooth rounded leather one-eighth inch in diameter. The electric current is passed through an Edison lamp screwed under the table to reduce the velocity of the motion. With the lamp in place and the current cut down to the minimum the number of revolutions per minute is 55, and the temperature of the water is the same in all parts of the bath. The simplest contrivance of all is to make the water-wheel and upright shaft of wood, to be turned by hand.

In localities where the gas-pressure is exceedingly variable, Paul Murrill's gas-pressure regulator (at the left in fig. 63) will be found useful. This is made by Eberbach & Co., Ann Arbor, Mich. (see *Journal of Applied Microscopy*, Vol. I, p. 92, or *Centralb. f. Bakt.*, 1 Abt., Band XXIII, 1898, p. 1056.) The gas-pressure may be somewhat improved by simply passing the gas through a big bottle (see top of thermostat 311 in plate 8). The Anschütz normal thermometers, with long stem and scale divided into fifths, are very convenient for determining temperatures (fig. 65). They come in sets of seven, but may also be had separately. The most frequently useful are No. 1 (scale  $-15^{\circ}$  to  $+55^{\circ}$ ) and No. 2 (scale  $+45^{\circ}$  to  $+105^{\circ}$ ).

They cost 9 marks each when ordered direct from Berlin, and can be had without delay. Good American thermometers are made by Henry Green, New York.

With this open bath it is easy to keep the range of temperature down to 0.1 to 0.2 of a degree, and the writer has frequently exposed tubes for ten minutes without appreciable change in temperature. Temperatures may be read easily to 0.1 degree by means of a Zeiss aplanat lens magnifying six times (fig. 25), and should be recorded for each half minute during the exposure. Under no circumstances should exposures be made in water which is not agitated. Of course, for accurate reading the eye and the center of the lens must be level with the top of the column of mercury. The lens may be supported at the proper level on a grooved piece of cork. If possible the thermometer used should be compared with some standard instrument. If not, it should at least be compared with several other good thermometers in the same laboratory. The test-tubes are supported by perforated corks thrust into holes bored through a rectangular piece of hard, *heavy* wood.



Fig. 65.\*

The writer formerly made use only of the first four tests. It seemed hardly worth while to recommend that all bacteria be tested for the killing effect of cold, so long as we had nothing but the inconvenient and more or less inexact methods of salt and pounded ice or of ether and frozen CO<sub>2</sub>; but now that liquid air may be obtained at a small price in many of the larger cities, can be shipped long distances, and can be used with so little inconvenience, there is no good reason why the effect of freezing should not be determined in all cases, since in some instances it is likely to prove a valuable means of differentiation. The bacteria may be exposed in 5 cc. portions of distilled water or bouillon in block-tin test-tubes, or preferably in tubes of resistant glass, for standard periods, *e. g.*, one-half hour, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, etc. They may also be exposed to alternate freezing and thawing every fifteen minutes or thirty minutes until all are dead. To avoid endospores, the depressing effect of by-products, etc., young cultures should be used, and, of course, all should be of the same age and grown in the same medium, *i. e.*, bouillon cultures 24 hours or 48 hours old. The tests should be quantitative rather than qualitative. They may be made as follows: Into 5 cc. of sterile water or standard bouillon a carefully-measured quantity, *i. e.*, one loop, 5 drops, 1/2 cc., etc., of the culture is placed, stirred very thoroughly, and allowed some time for diffusion. To avoid zooglœæ, which form early in some species, and to reach more uniform measurements, it is recommended to take the loop from a bouillon culture rather than from agar or other solid media. After sufficient time has elapsed for uniform diffusion, six Petri-dish poured plates are made from each of the inoculated tubes. The plates should be of the same diameter (area of 60 sq. cm.). The amount of agar used for each plate should be 10 cc., and the amount of infec-

\*FIG. 65.—Anschutz normal thermometer with degrees divided into fifths (Centigrade scale). For use in thermal death-point tests. About three-fourths actual size.

tious material used should be the thinnest obtainable film of fluid across a carefully-measured 1 mm. loop, so as to avoid crowding the plates. The same loop should be used in all cases, and it should be dipped into and out of the fluid always in the same way. After pouring, set the plates on a perfectly level spot (fig. 66), until the agar has hardened. If the work has been well done, there should develop an approximately uniform number of colonies in each plate. The tubes of inoculated water or bouillon are then immediately lowered into the liquid air and exposed to it for the predetermined time, after which six additional Petri-dish poured plates, of the same size and inoculated in the same way, are made from each tube for comparison with those prepared before the exposure. The tubes may be thawed out by exposure to the air for three minutes and then to tap-water for five to seven minutes. The exposures are best made in Dewar glasses (fig. 67). When the exposures are long, a loose tuft of absorbent cotton should be placed in the mouth of the glass, or it should be covered with a hair-cloth cap, to prevent excessive



Fig. 66.\*

evaporation. Under these conditions the air remains liquid for a number of days. At first the temperature is about minus 190° C., rising gradually to minus 180° C., since the nitrogen evaporates somewhat faster than the oxygen. The glasses are fragile and should be handled carefully, especially when filled with the air. As long as they contain liquid air it is safer to keep them in their containing-case, packed about with cotton or felt. One should be careful to avoid cracking the inner wall of the glass, as might happen by dropping some hard substance into the receptacle, otherwise an explosion will occur, the space between the two walls of the Dewar glass being a very perfect vacuum.

When the exposures are made in block-tin tubes, the culture should be frozen at once on pouring into the tube and the second set of plates should be made as soon as the fluid has thawed, *i. e.*, within about ten minutes, for which purpose the culture should be poured out into a glass tube, otherwise complications due to

\*FIG. 66.—Leveling (nivelling) apparatus for use in making poured plates. About one-third actual size.

the germicidal action of the metal might arise. In no case should the cultures be incubated in the tin tubes. When exposures are made in test-tubes of resistant Jena glass, the cultures must be lowered into the liquid air gradually, the fluid being frozen from the bottom upward to avoid cracking the tubes. It requires about four minutes to properly freeze a culture in a glass test-tube. Large volumes of culture media should not be lowered into the liquid air, as it is wasteful, the air boiling away rapidly. The writer began his experiments with block-tin tubes, as shown in fig. 67, but now uses tubes of Jena glass. The latter crack occasionally in spite of care.

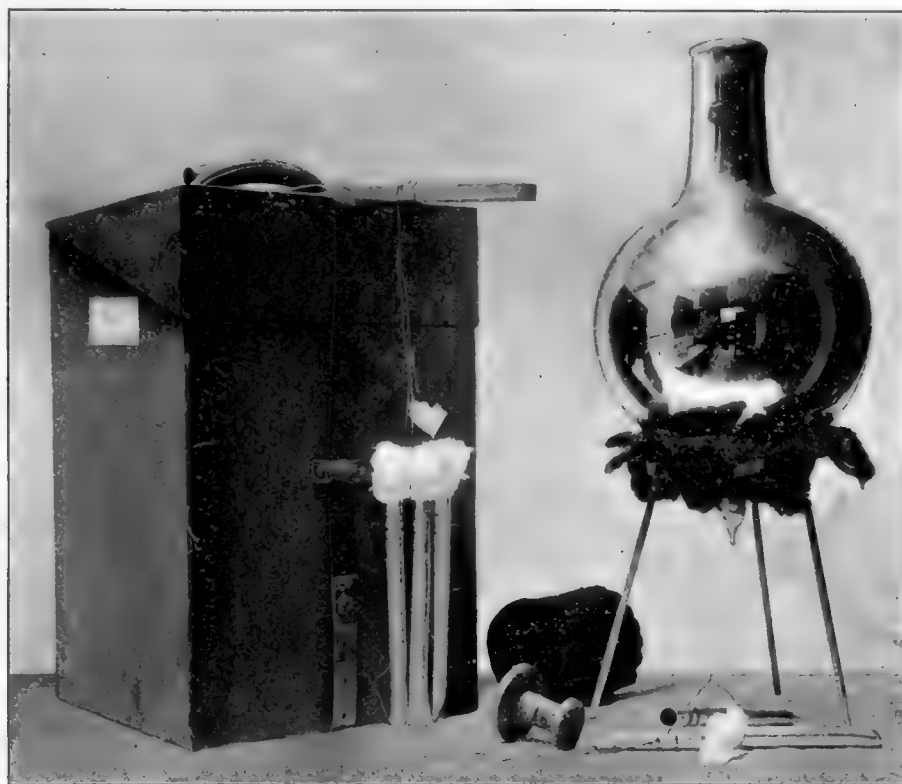


Fig. 67.\*

For very rapid freezing the amount of fluid in the tube may be reduced to 1 cc. Liquid air in Dewar glasses, and compressed oxygen, hydrogen, and carbon dioxide (?) in steel cylinders may be had from the Eagle Oxygen Company, Incorporated, 121 West Eighty-ninth Street, New York City. The tanks of compressed gases may be bought or rented. The following sizes may be had: Fifty gallons (280 pounds pressure per square inch); 100 gallons (240 pounds pressure); 150 gallons (225 pounds pressure); and 200 gallons (280 pounds pressure). Cylinders may also be had with the gas under much greater pressure. The cost of the oxygen is  $2\frac{1}{2}$  cents

\*FIG. 67.—Dewar glass for liquid air, and block-tin test-tubes used in first low temperature experiments with bacteria. About one-sixth actual size.

per gallon. The wrought-steel cylinders cost about \$10 each. A good quality of resistant-glass test-tubes may be had from Greiner & Friedrichs, Stützerbach, Germany. One sort has a faint-blue longitudinal stripe blown into the glass, another kind has the letter "R" etched on the upper part of each tube. Tubes without any distinguishing mark should not be purchased, as they are likely to become mixed with ordinary non-resistant tubes. The cost of these tubes, duty free, is about \$16 per thousand. Good Petri dishes may be obtained from the same firm, and also from E. H. Sargent & Co., Chicago.

The temperature demands of bacteria are extremely variable. Whole groups of them are able to live under conditions which would be impossible for the higher

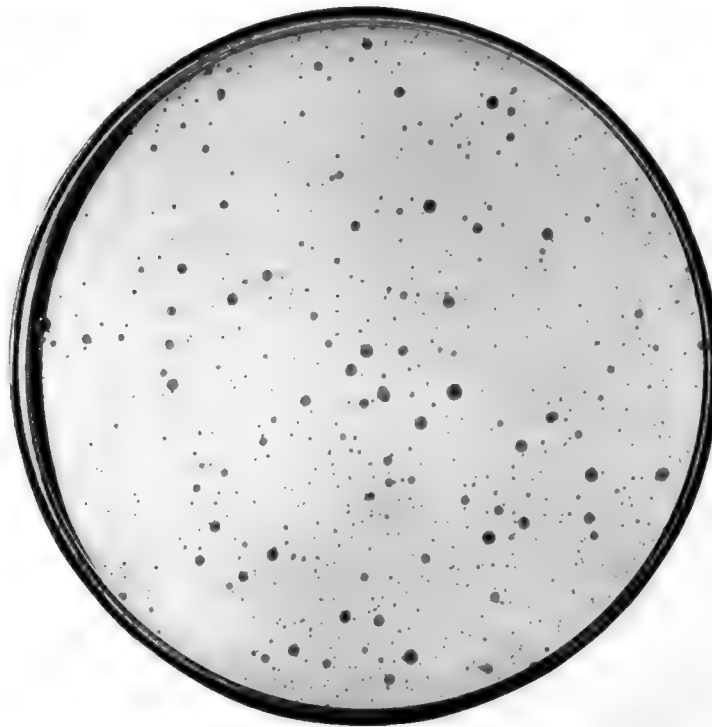


Fig. 68.\*

plants and animals. Many of the northern forms, especially those which grow in water, are adapted to low temperatures. The organisms of dung-heaps and thermal springs, and the tropical forms, often grow at high temperatures.

For a very few species it has been known that prolonged freezing or repeated freezing and thawing destroys the weaker individuals and finally all. (See Bibliog., XXXIII, especially papers by Sedgwick & Winslow, and by Park; consult also an earlier paper by Prudden, Bibliog., XLVI.) For the bacteria as a whole, however, it has been assumed that ordinary freezing or even very intense cold simply inhibits

\*FIG. 68.—Petri-dish poured plate of *Bacillus tracheiphilus*. The 10 cc. of nutrient agar was inoculated with a carefully measured loop of a fluid culture. The fluid culture was then exposed in block-tin test-tubes to the temperature of liquid air, after which another plate (fig. 69) was made.

growth for the time being. Such statements have been based on certain qualitative tests and do not tell the whole truth. In the writer's experiments with liquid air great differences have been detected, the reduction by exposure for one-half hour varying from 15 per cent, or less, to 90 per cent, or more, according to the species tested. Fully 50 per cent of many sorts, grown in bouillon, are destroyed by a single short exposure (see figs. 68 and 69). Query: Is intense cold any more harmful to bacteria than simple freezing? Are young or old cultures most susceptible? Are they killed by the rupture of the cell-wall due to the formation of ice-crystals, or simply by the abstraction of water? Why do some resist several freezings? Can endospores be killed in this way? Consult '01, d'Arsonval (Bibliog., XXXIII) and

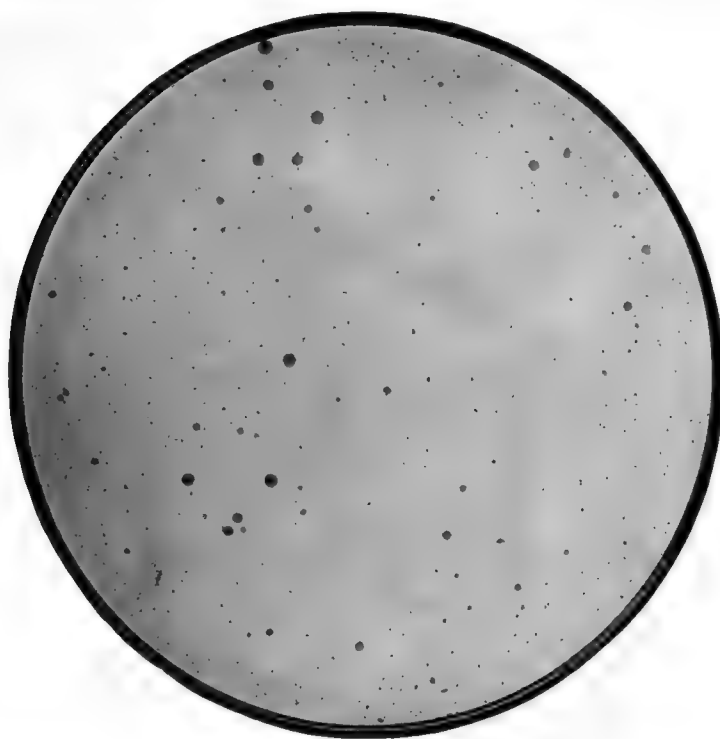


Fig. 69.\*

Smith & Swingle, the Effect of Freezing on Bacteria, Proc. Sixth Ann. Meeting Soc. Am. Bacteriologists, December 27, 1904; Science, N. S., Vol. XXI, 1905, pp. 481-483. For opposing views see '02, Macfadyen, Bibliog., XXXIII.

Live steam acts upon the growing bacteria very quickly. All bacteria not in spore form, or in some other way protected from the direct action of the heat by what surrounds them, are promptly destroyed by steam heat at 100° C., an exposure of a minute or two being ample, except, possibly, in case of some of the thermo-

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\*Fig. 69.—Same as fig. 68, but made after exposure for twenty hours to liquid air. Number of colonies reduced two-thirds. Exposed in test-tubes of Jena-glass for one-half hour, the reduction was nearly as great, *i. e.*, over 50 per cent. In this latter case the agar plates were incubated 7 days at 30° C., before the count was made.

philic species. Usually even the most resistant spores, if freely exposed, are destroyed by one to two hours exposure to  $150^{\circ}\text{C}$ ., of dry heat, or by thirty minutes exposure on each of three consecutive days to streaming steam at  $100^{\circ}\text{C}$ . Some very resistant spores have survived a single steaming or boiling of five or six hours duration (eight hours in one of Tyndall's experiments), and it is not unlikely that some slowly germinating sorts may be able to resist discontinuous steamings for three days. It is possible also that there may be some sorts able to germinate and again assume a resistant spore form in less than twenty-four hours although this is not probable. Some spores are destroyed by a short boiling at  $100^{\circ}\text{C}$ ., and all spores are quickly destroyed by steam under pressure, *i. e.*, in an autoclave. A

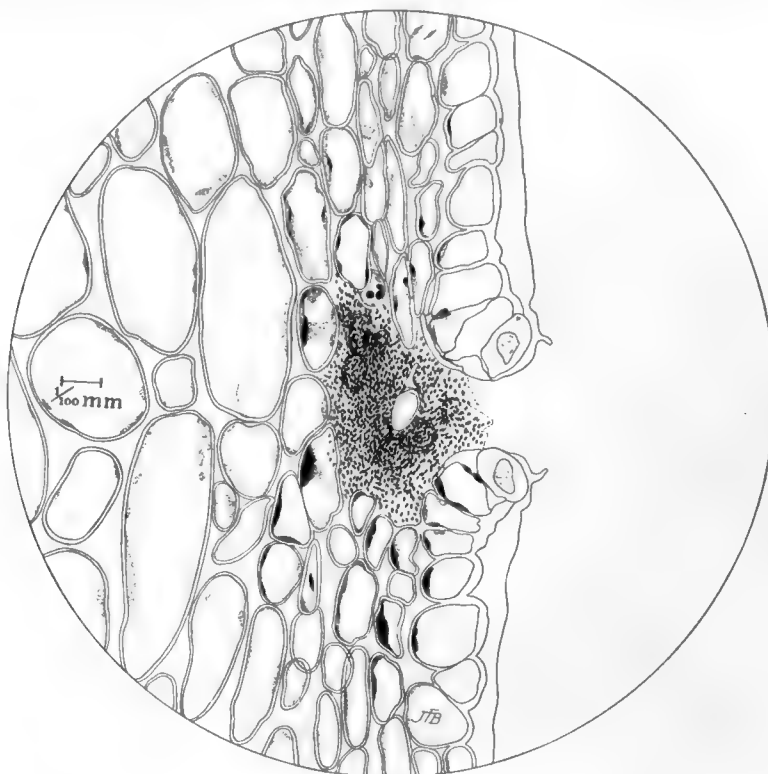
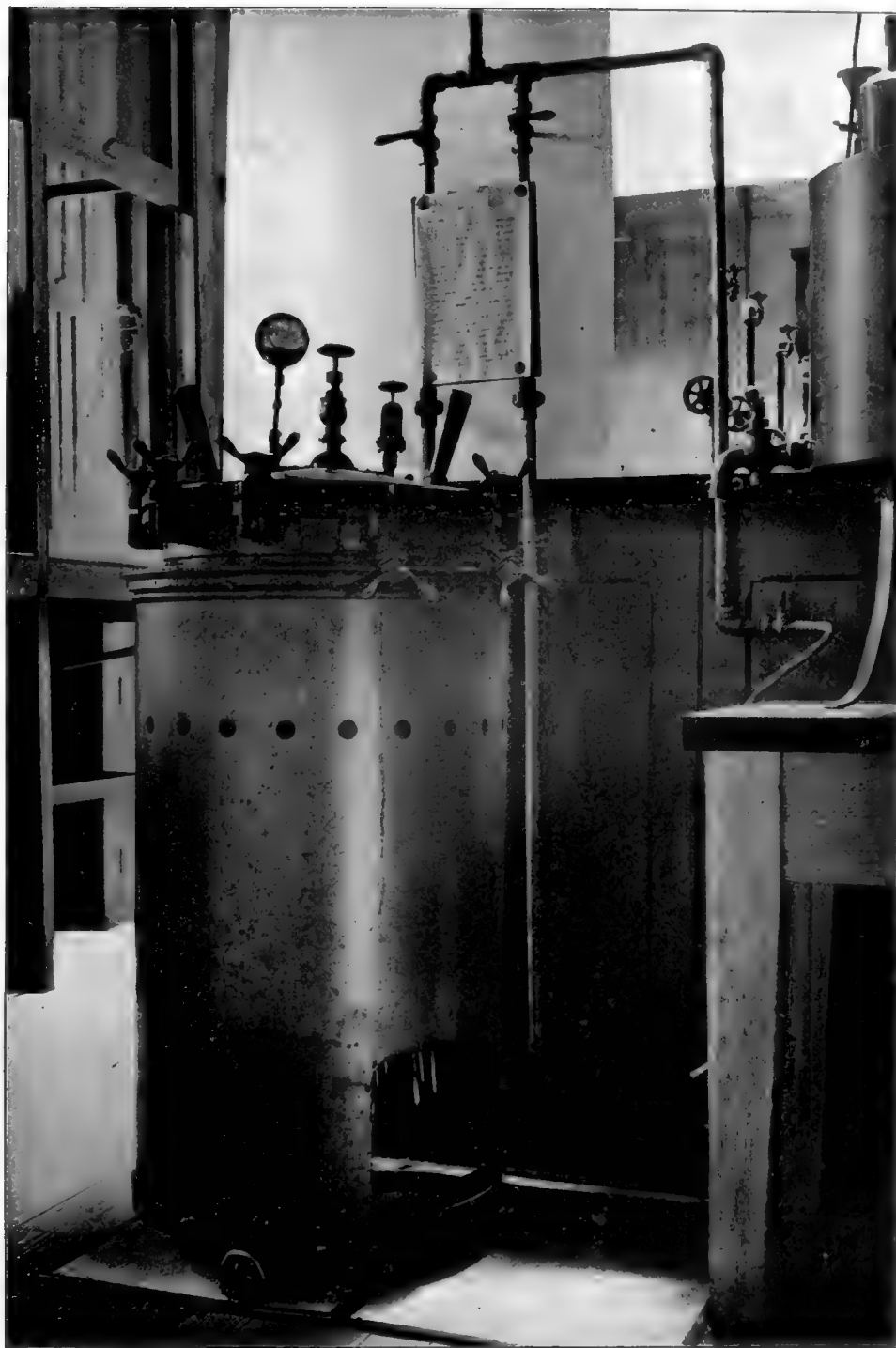


Fig. 70.\*

temperature of  $110^{\circ}\text{C}$ . for ten or fifteen minutes is sufficient. Exposure of media to higher temperatures and for longer periods should be carefully avoided. It must be remembered, however, in using autoclaves, that all of the air must be replaced by steam before the apparatus is closed, otherwise the temperature to which the medium is exposed will not correspond to that indicated by the pressure gage. The most convenient autoclaves known to the writer are the large sizes of the

\*FIG. 70.—Earliest stage of fruit spot on green plums, due to *Bacterium pruni* (Erw. Sm.). The bacteria have entered through the stoma. They disappear farther in, and also a few micra to either side of this stoma, as shown by an examination of the serial sections. Material fixed in strong alcohol, infiltrated with paraffin, and cut on the microtome in series. Section stained with carbol-fuchsin and drawn directly from the microscope with the aid of a camera lucida.





Chamberland autoclave.

Heat is applied to the bottom by means of a double ring of Bunsen burners. No wrench is required for fastening on the top. About one-eighth natural size.



pattern designed by Chamberland and made by the Maison Wiesnegg (P. Lequeux), Paris, France, the steam being generated by gas (plate 9). The steam gage is at the left; in the middle is the valve through which the hot air is allowed to escape when the instrument is warmed up; at the right is the steam safety-valve. The temperature is manipulated by regulating this valve. By leaving the vent open the apparatus may be used as an ordinary steam sterilizer. It may also be used as a distilled-water apparatus by attaching a condenser to the exit pipe of the middle vent, but such water must not be used for culture media. A very good autoclave is also made by the Kny-Scheerer Co., New York. Harding recommends for autoclaves the use of steam from the engine-room boiler. This is convenient, provided one can always have steam ready during the summer months. An autoclave, like a steam boiler, which it is, must be watched carefully if it is not some time to explode from excess of heat or lack of water. Each time before use one should see that the apparatus contains sufficient water.

Soils are rather difficult to sterilize. They may be spread in thin layers and dry-heated for several hours at  $150^{\circ}\text{C}$ ., or may be heated in the autoclave for an hour under a pressure of two atmospheres, taking care to drive all the air out of the soil before closing the apparatus. It is not likely, however, that soils can be treated in this way without undergoing certain physical and chemical changes. Small pots of soil may be heated in the steamer at  $100^{\circ}\text{C}$ . for two hours on each of five successive days.

The reason for preparing all media in the autoclave, or by heating in the steamer at  $100^{\circ}\text{C}$ . on three successive days (the ordinary way), is because we are never certain in what particular case resistant spores may be present. One short steaming is often sufficient to sterilize media prepared in a cleanly way, as every bacteriologist knows who has had much experience, but now and then, in spite of all care, resistant spores will find their way into culture media, and for this reason it is best in all cases (especially in teaching students) to adhere to a routine of three steamings. Large masses of fluid (beakers, flasks) require longer steamings than test-tube cultures. The writer gives double time, or triple time. Discontinuous boiling as a means of sterilization was introduced in 1877 by Tyndall, who well says respecting the sterilization of liquids: "Five minutes of discontinuous heating can accomplish more than five hours continuous heating."\*

Most plant-pathogenic bacteria of temperate and cold regions have a lower optimum and maximum temperature for growth and a lower thermal death-point than species pathogenic to warm-blooded animals. The maximum temperature for growth is usually at or below  $36^{\circ}\text{C}$ . We should not, however, expect this to be true of bacterial plant parasites in tropical and sub-tropical regions, about which, however, little is known beyond the mere fact that such parasites occur. Savastano states that the optimum temperature for the olive-knot organism, which is said to be more prevalent at the southern than at the northern limit of olive-growing,

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\*This method appears to have been known to housewives for a much longer time. In Dr. Samuel Johnson's Dictionary (first Am. from eleventh London ed.) I find the following definition: "Biscuit, A kind of hard, dry bread made to be carried to sea. It is baked for long voyages four times."

*i. e.*, commonest in southern Italy, Sicily, and Algeria, lies between  $32^{\circ}$  and  $38^{\circ}$  C. In my own experiments with this organism, obtained from olive trees in California, I have found its maximum temperature to be above  $35^{\circ}$  and below  $37.5^{\circ}$  C. The optimum temperature of *Bacterium solanacearum*, which is very destructive to potatoes and tomatoes in the southern United States, is probably about  $35^{\circ}$  C.—at least it grew readily and remained alive for a long time in bouillon kept at  $37^{\circ}$  C. Its maximum temperature is  $39 + ^{\circ}$  C. *Bacillus carotovorus*, one of the best known of the soft-rot organisms, grows well in the thermostat at  $33^{\circ}$  to  $34^{\circ}$  C. Its maximum temperature is at  $39^{\circ}$  C. or slightly below (Jones). *Bacillus aroideæ*, whose temperature relations were recently studied carefully by Townsend, has a maximum

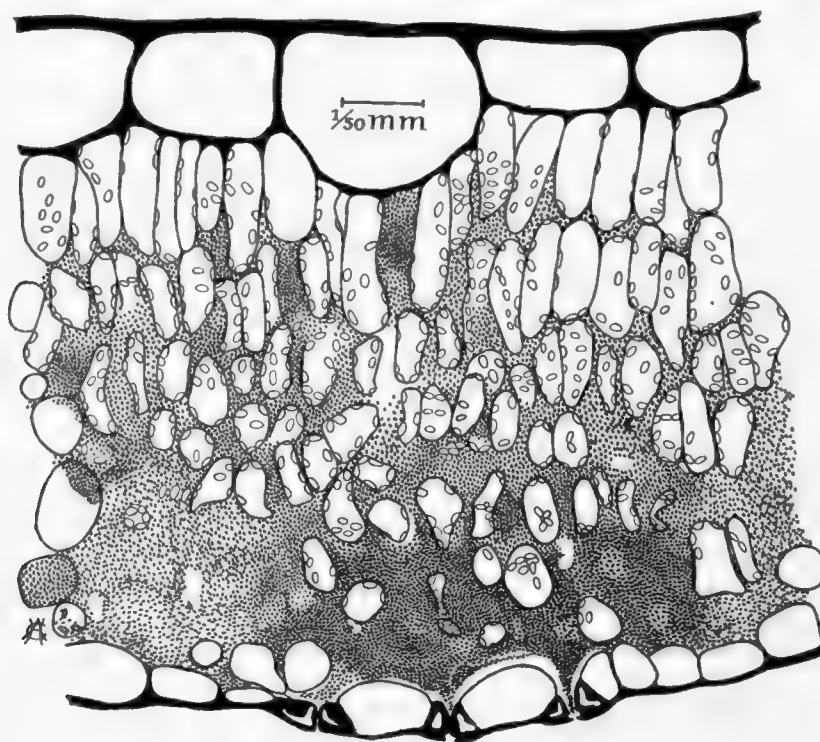


Fig. 71.\*

temperature of  $41^{\circ}$  C. A temperature of  $40^{\circ}$  C. retards growth, but does not prevent it. This organism was isolated from calla-lily corms, but is capable of causing a soft rot in potatoes, carrots, turnips, and many other plants (fig. 102). The maximum temperature of *Bacillus oleraceæ*, recently described by Harrison, is said to be about  $42^{\circ}$  C. This causes a soft rot of cauliflower.

The range of temperature suited for the growth of particular bacteria varies greatly. Some species are able to grow through a range of  $50^{\circ}$  C. Many tolerate a range of only about  $30^{\circ}$  C. Certain animal-pathogenic forms have through long subjection to a peculiar environment become restricted to a still narrower range.

\*FIG. 71.—*Bacterium pruni*. Early stage of a leaf-spot in the plum. The small spot was water-soaked in appearance, but it had not yet collapsed. The bacteria, which are most abundant in the mesophyll, undoubtedly entered the leaf through the stomata, three of which are shown in the section. Material treated as in fig. 70. Section drawn with the aid of an Abbe camera. It represents as nearly as possible one plane.

Some bacteria grow well only in the cool box, others only in the thermostat at blood-heat or at higher temperatures,—temperatures elevated enough to quickly destroy the unprotected protoplasm of the higher plants and animals. Few of the bacteria commonly studied will grow at temperatures much above 40° C., but this by no means expresses the whole truth.

The *lowest temperature* at which growth will take place ranges in different species all the way from 0° C., and probably a few degrees below (certain salt-water bacteria) to + 40°, + 50°, + 56°, and even + 60° C. (certain thermophilic species found in dung-heaps, hay-mows, silos, hot springs, etc.). The *highest temperature* at which growth will take place ranges from as low as 30° C. (and probably lower\*) to as high as 75°, or 80° C., or even 89° C., according to Setchell. Higher temperatures have been recorded, but I have here used only those determined with care in the exact places frequented by the bacteria. This will be better appreciated if it is remembered that a temperature of 60° C. (140° F.) can be endured by the fingers only a few seconds, while 70° C. (the optimum for some of these species) is intolerable to the hand even for the shortest period. It seems incredible, on first thought, it is so opposed to our customary observations, that any organism whatsoever should be able to live at a temperature only 11 degrees below the boiling point of water. Nevertheless, protoplasm is an extremely adaptable substance, and it is conceivable that some organisms might grow at a temperature considerably higher.

The thermal death-point (10 minutes exposure) ranges from 43° C. for *Bacillus tracheiphilus*, the lowest yet recorded,† to temperatures only a few degrees under the boiling point (100° C.). For many species the thermal death-point lies between 50° and 60° C. Russell & Hastings have recently discovered in milk a *Micrococcus* whose thermal death-point is 76° C.

As the upper and lower thermal boundaries of growth are approached some functions are extinguished in advance of others; *e. g.*, pigment production, pathogenicity, and sporulation disappear considerably in advance of loss of power to reproduce by fission.

#### OTHER HOST PLANTS.

Plants of related species, genera, and families should be tested. If the disease appears to be new to literature, it is also especially important to inoculate those plants which have been reported to be subject to bacterial disease and the nature of which disease is still in doubt. Many facts of scientific and economic interest will be brought to light in this way, and now and then the experimenter may be able to clear away some of the fog which, owing to the uncertain and contradictory statements of a majority of our plant pathologists, still hangs over the origin and nature of most of these diseases.

Some plant pathogenes appear to be quite narrowly restricted. They attack only one host plant, or at most a few hosts belonging to related species or genera. Others, particularly some of the soft-rot bacteria, attack many kinds of plants belonging to widely different families. The history of pear-blight, however, shows us that

\*Since this was written Molisch states (l. c., p. 93) that gelatin cultures of his *Bacterium phosphoreum* were dead at the end of 48 hours when exposed to a temperature of 30° C. The maximum temperature of this organism is said to be about 28° C.

†Very recently Marsh has found a fish parasite which is said to have a thermal death-point of 42° C. (See VI, Bibliography of General Literature.)

the restriction of an organism to a single host-plant may be only an inference based on insufficient observation rather than an actual fact. After a time the apple and quince were added to the pear as host-plants, and now we may add also the plum and the loquat.

#### PATHOGENIC OR NON-PATHOGENIC TO ANIMALS?

If the organism will not grow in the thermostat at 37° C., or grows only feebly, as is the case with many plant parasites, it may be assumed to be non-pathogenic to animals with warm blood. Only those organisms which grow readily in the thermostat at 37° C., and which closely resemble animal-pathogenic forms or which are suspected of causing some particular disease of animals, need be tested

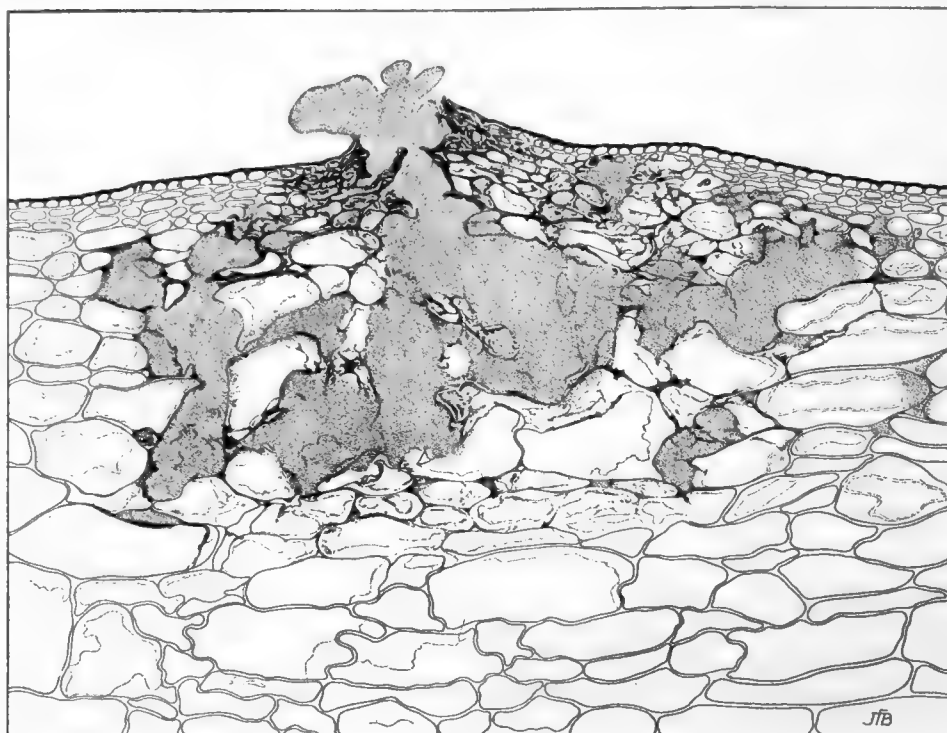


Fig. 72.\*

by animal experimentation for economic purposes. In general, it is best to leave this part of the work to the animal pathologist, for the same reason that the more abstruse chemical problems are turned over to the chemist.

All of the plant-parasitic bacteria, so far as tested, have turned out to be non-pathogenic to warm-blooded animals, but it is not unlikely that some exceptions may be discovered.

Another question, of special interest to animal pathologists, arises here, namely, whether forms known to be pathogenic to animals and especially to man are ever

\*FIG. 72.—*Bacterium pruni*. Vertical section through a green plum fruit (var. Hale) showing bacterial cavities and the escape of the organisms through the ruptured stoma. In this case beyond doubt the central stoma is the one through which the infection originally took place. Drawn from a photomicrograph. The material was fixed in alcohol, infiltrated with paraffin, cut on the microtome, and differentially stained.

harbored by plants. Of those known to cause animal diseases none have ever been found naturally present in plants, but some of them, such as the typhoid bacillus, the anthrax organism, etc., have been shown to live for a number of days or weeks when injected into various living plants, and in some instances have been found to multiply a little in the vicinity of the wounds. In general, their life is short in such situations, they do not penetrate far into the tissues, and they are

manifestly on the defensive. If they can do no better when injected into vegetable tissues in enormous quantities, it seems rather unlikely that under ordinary natural conditions they would find their way into plants so as to make them dangerous for food. In this connection the reader is referred to Volume II, where this subject is discussed more fully. More danger is likely to result from pathogenic organisms carried on the surface of plants, especially on salads and fruits which are not cooked. In times of the general prevalence of typhoid fever, cholera, or the bubonic plague, the writer for one would certainly prefer to forego salads and to eat only freshly cooked vegetables. The danger from such foods in time of epidemics is very great, especially in localities where ditch-water is frequently sprinkled on the vegetables to freshen them, *e. g.*, in parts of southern Italy.

Most saprophytes when injected into living plants behave in the same way as the animal parasites, *i. e.*, they either die at once or maintain a pre-



Fig. 73.\*

carious existence for some weeks in the vicinity of the wound and then succumb. The writer has made many experiments, with negative results. The most extensive published series of experiments are those of Zinsser (Jahrb. f. wiss. Bot., 1897). To get a particular disease, the parasite must be used and not some other organism. This the writer has observed over and over again. This statement holds good with plants the same as with animals. In case, however, of the less typical plant diseases (soft rots) various members of a group of closely related organisms may produce essentially similar phenomena. This is paralleled, however, in certain of the less typical animal diseases.

\*FIG. 73.—Seedling sweet-corn plant extruding water from its leaf-tips. Most of the infections by *Bacterium Stewarti* take place during this stage of growth, the bacteria passing down the leaf through its vessels and entering the stem through the lower nodes. Natural size.

## ECONOMIC ASPECTS.

The economic aspects may be considered under four heads: (1) Losses; (2) Natural methods of infection; (3) Conditions favoring the spread of the disease; (4) Methods of prevention.

In the United States Department of Agriculture and in our State Experiment Stations, naturally, much stress is laid on economic considerations, especially on 2, 3, and 4. A knowledge of 2 and 3 will frequently lead to some simple and effective means of prevention.

## LOSSES.

It is desirable that there should be made from time to time a careful estimate of the losses caused by each particular disease, not only as a warning to farmers, fruit-growers, market-gardeners, and florists of the existence of these dangers, but also as an aid to legislatures and governments in deciding how much money may be judiciously appropriated for the scientific investigation of these problems. Pathologists are urged to make and publish such records. It is perhaps unnecessary to add that the determinations should be reasonably accurate, otherwise it were much better not to make any records. Occasionally, when diseases are widespread and destructive, so that depreciation of land values and the hostility of a community might result from great publicity, the pathologist may have to consider discretion the better part of valor and refrain from publishing, but in this event he should not fail to make full records which may subsequently be published or at least consulted. What we need and must finally have is a large body of accurate statistics, covering a series of years, many localities, and many diseases. To make these statistics most useful, certain meteorological data should be collected in the same localities. To be of most service this data concerning the weather should be recorded by the pathologist himself, who will be better able than anyone else to note down just those things likely to influence the host-plants favorably or unfavorably. Some of these things

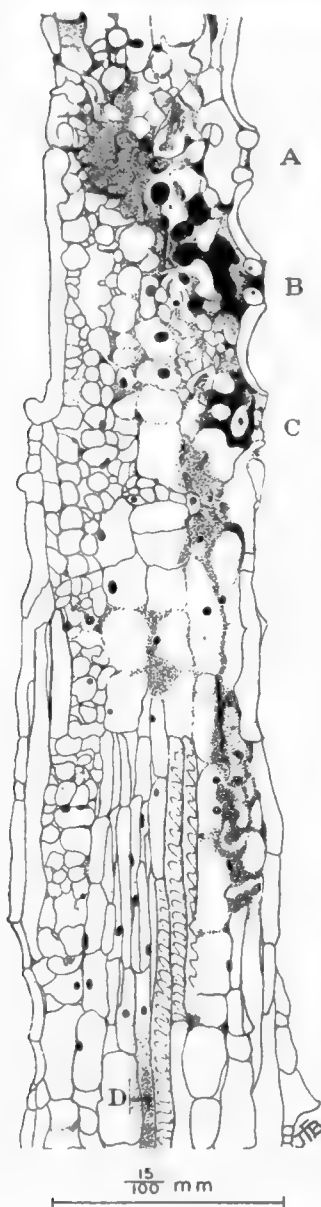


Fig. 74.\*

\*FIG. 74.—*Bacterium Stewarti* (Erw. Sm.) attacking sweet corn (*Zea mays*). The section was cut from the extreme upper part of a seedling leaf which was fixed in strong alcohol six days after placing the bacteria on its tip. At the time of inoculation water was extruding from the leaf-tip, as shown in fig. 73. This figure represents a longitudinal vertical cut. The dotted and heavily shaded parts show the location of the bacteria which have entered through the ordinary stomata and have not yet penetrated the vascular system, although in places, as at D, they are close to the spiral vessels. At A, B, and C are three stomata. The substomatic chamber under A is free. B, with its surroundings, is shown more highly magnified in fig. 75. Drawn with help of the Abbe camera.



are cloudy weather (especially if prolonged), sunny weather, frequent or excessive fogs or dews, amount of rainfall, and frequency of rainfall, snowfall, hail, excessively hot weather, cold spells and frosts, droughts, daily maximum and minimum temperature, prevalence of special diseases correlated with special peculiar conditions, absence of other diseases, etc.

#### NATURAL METHODS OF INFECTION.

Under this heading the student should be on the watch for transmission of the disease through fungous or insect injuries, by mollusks, by birds or quadrupeds, and by the hand of man. Man contributes to the spread of diseases in various ways,

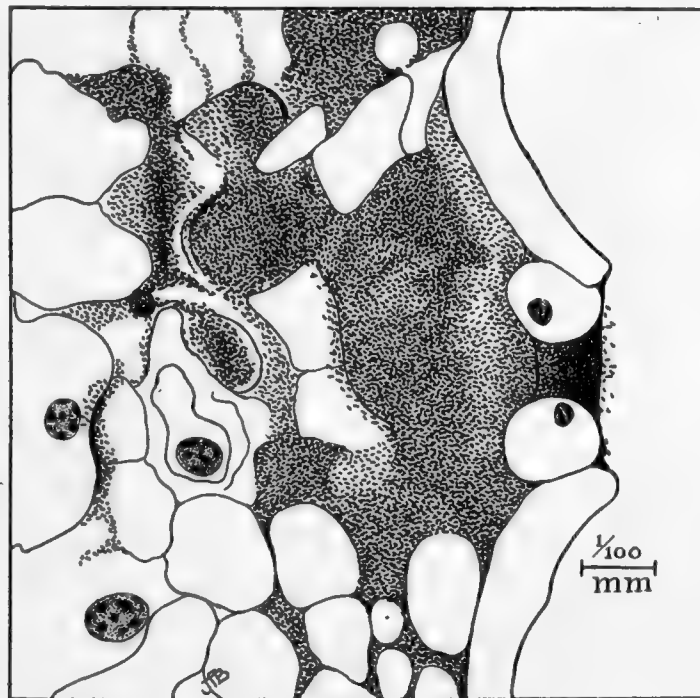


Fig. 75.\*

*e.g.*, by neglect to remove diseased plants, by use of infected knives and other tools, by the introduction of infected seeds, or manures, or soils, or water, and by subjecting his plants to a variety of depressing and unwholesome conditions.

A great variety of parasites find their home in the earth, the top crust of which swarms with bacteria and fungi. Such parasites are frequently introduced from one locality to another in infected soils adhering to wagons and other farm tools, to the feet of men and animals, to the roots of transported plants, etc. The soil is a living thing and it should not be transported even from one field to another on the same

\*FIG. 75.—*Bacterium Stewartii* filling the substomatic chamber and pushing out into the deeper tissues of a maize leaf. The result of an inoculation made by placing a small quantity of a pure culture on the tip of a sweet-corn leaf in the seedling stage. For orientation see fig. 74. The globose bodies are nuclei, which are not enlarged (?).

farm without due consideration of what may happen. Certain bacterial diseases might be distributed very readily in this way and good fields rendered worthless for certain crops.

The parasite may gain entrance to the plant through wounds (plates 2 and 4 and fig. 8) or by way of the stomata (figs. 70 to 75), lenticels, water-pores (figs. 76 to 79), and nectaries. In recent years the writer has discovered a number of very characteristic infections by way of the stomata and the water-pores, which are only modified stomata, *e. g.*, in cabbage, mustard, plum, bean, soy-bean, cotton (fig. 80),

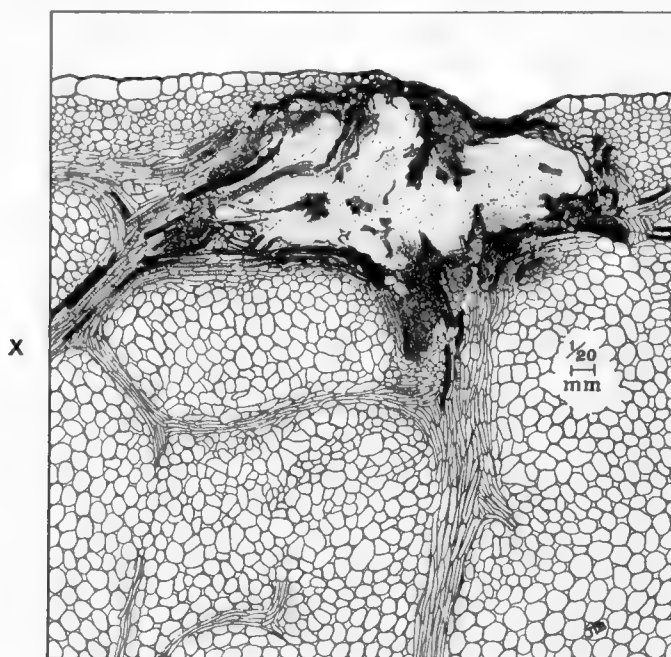


Fig. 76.\*

pelargonium, larkspur, broomcorn, sorghum, maize, cucumber, etc. Pear-blight affords one of the most striking examples of wholesale infection by way of the nectaries. The wilt of cucurbits affords an equally good example of infection through wounds—namely, leaf-injuries due to beetles.

\*FIG. 76.—*Bacterium campestre*. Section of a cabbage leaf parallel to the surface and near the margin, showing the result of infection through the water-pores. The tissues are browned and destroyed. Immediately under the leaf-serrature a cavity has formed and the bacteria have begun to penetrate into deeper parts of the leaf by way of the spiral vessels, not all of which are occupied. This figure is slightly diagrammatic, but only to the extent of omitting the protoplasmic contents of the parenchyma cells and of introducing six occupied spiral vessels which belong to the next section in the series. No spiral vessels are visible in the lower part of the section because the knife passed just below them. Material collected on Long Island, July 16, 1902, and fixed in strong alcohol. The spirals here shown are a little too densely occupied by the bacteria to make a good drawing under the oil-immersion objective, but a little farther in (beyond X) they are less abundant and entirely satisfactory for this purpose.

## CONDITIONS FAVORING THE SPREAD OF THE DISEASE.

The conditions favoring the spread of diseases may be wholly telluric, such as high temperature, unusual drought, cold weather, fogs, heavy dews, and excessive or continuous rainfall. These diseases may be favored by lack of natural drainage, or may be brought on by a variety of causes which are largely within the control of the grower, such as selection of improper varieties, *i. e.*, very susceptible ones, overcultivation, storage at too high temperatures (in case of cabbage and root crops), the use of infected soils, or manures, or seeds, or plants, and, especially in hot-houses, by the mismanagement of water and heat, and by the neglect to destroy the first diseased plants that appear and such transmitters of disease as insects and slugs, which frequently abound in hot-houses.

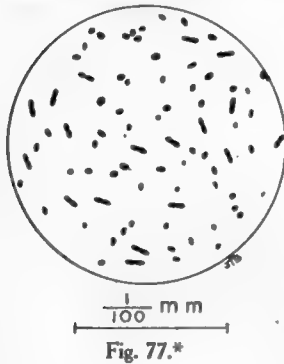


Fig. 77.\*

## METHODS OF PREVENTION.

In case of certain diseases, copper fungicides have been found useful, *e. g.*, in walnut bacteriosis and some of the leaf spots, but in general we know as yet very little about bactericidal treatments. In the early stages of an outbreak some of these diseases may be controlled by extirpation of the affected parts, or by the removal of whole plants as soon as they show signs. Also, if possible, the common carriers of infection should be eliminated. Finally, one should not forget that the substitution of resistant varieties for susceptible varieties is one of the most hopeful methods for disposing of certain of these vexatious diseases. Whenever anything specially noteworthy has been discovered in the way of treatment it will be mentioned under each particular disease.

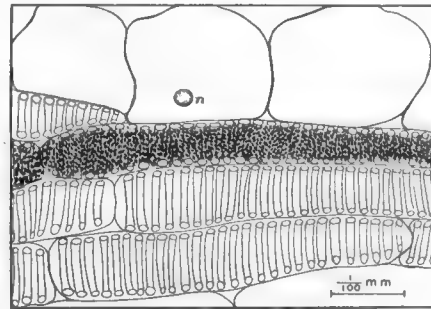


Fig. 78.†

\*FIG. 77.—*Bacterium campestre* from the cavity shown in fig. 76, illustrating water-pore infection of the cabbage.  $\times 2,000$ .

†FIG. 78.—*Bacterium campestre* occupying a spiral vessel in a cabbage leaf near a group of infected water-pores. The tissues to the right and left of this vessel, and also above and below it (slide 223 a3, 18.6 by 9.7), are entirely free from bacteria. The body of the leaf and all its inner tissues up to within a few millimeters of the leaf-tooth, and also the outer surface of the leaf up to the water-pores, are sound. On the contrary, an unbroken bacterial occupation can be traced from this vessel outward to the water-pore region. The bacteria in this vessel are also less abundant than in those nearer to the group of water-pores, *i. e.*, its occupation is of more recent date. Even if there were no other evidence of infection by way of the hydatodes than that afforded by this vessel, the presence of the bacteria in it under the circumstances mentioned points conclusively to marginal (water-pore) infection as their only possible source. The position of this vessel is in a small vein a little below and at the left of X in fig. 76. Its distance from the left margin of the bacterial cavity is one field of the 16 mm. Zeiss objective with the 12 comp. ocular. Its distance from the sound leaf margin is two-thirds the diameter of such a field. A nucleus is shown at n.

## GENERAL CONSIDERATIONS.

## LOCATION OF THE LABORATORY.

If possible, the laboratory should be in a clean building in the middle of a green lawn. If it must be in a crowded and dirty city it should be on an upper floor, as far removed as possible from the dust of the street and from the tramp of feet. It ought not to be located on streets filled with the dust of heavy traffic. If a ground-floor or basement room in a dirty locality is the only available place, then the air which enters the room should be filtered through absorbent cotton. A south front is desirable for the mounting of a heliostat and for other photographic purposes; a north light is desirable for microscopic use, if one is to work at the instrument continuously. By arranging one's time according to the position of the sun, the light from east or west windows may be used to advantage five or six hours a day, which is quite long enough to fatigue ordinary eyes. The writer has managed to

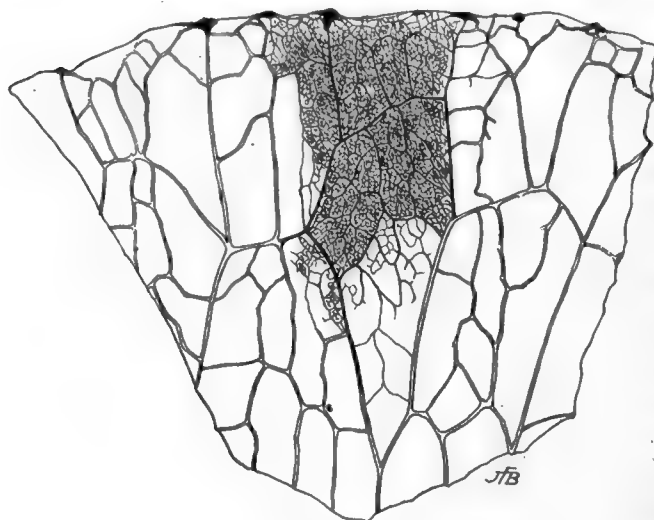


Fig. 79.\*

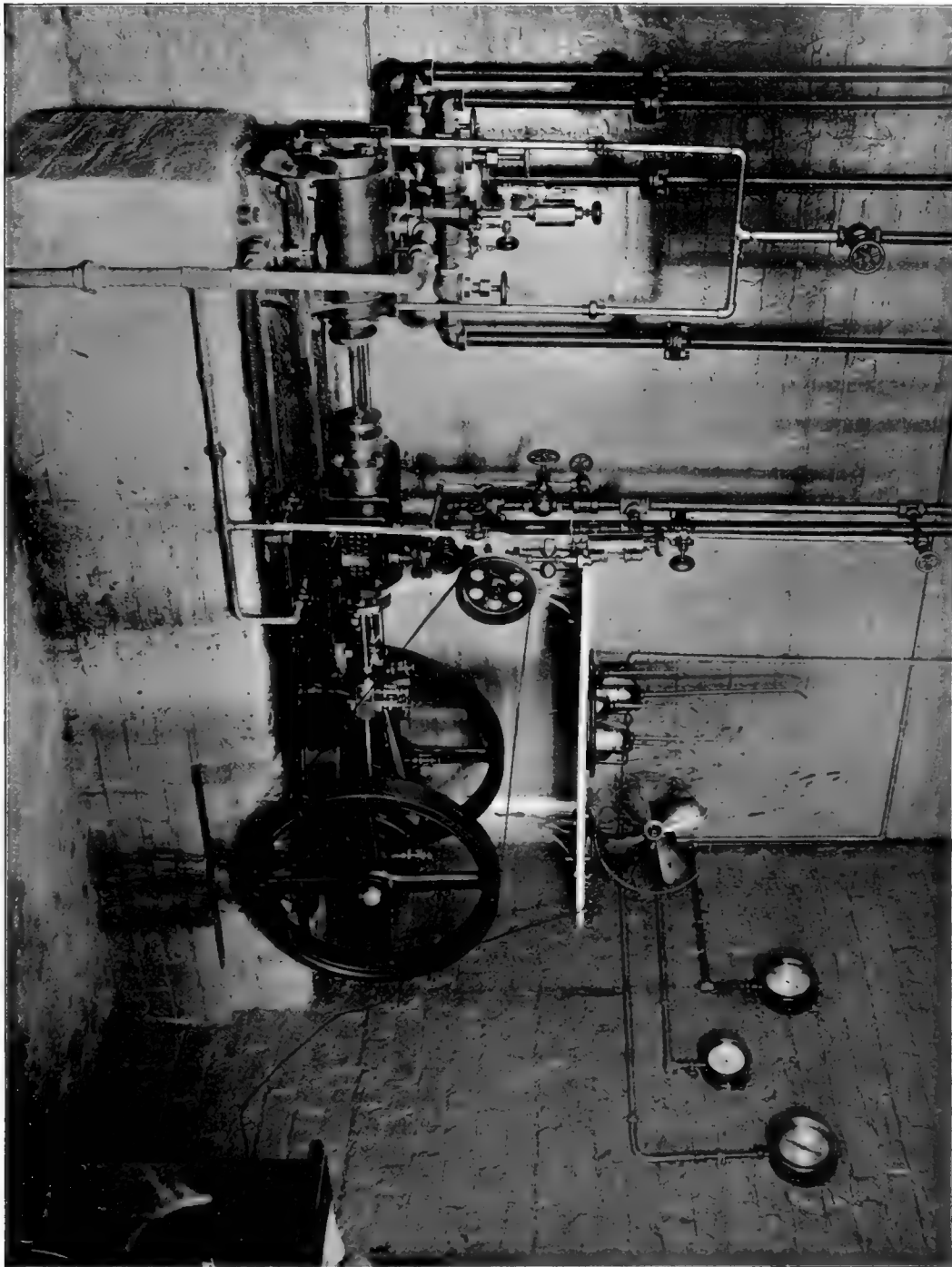
get along very well without north light for the last ten years. If one decides to use with the microscope only artificial light, such as that of the Welsbach burner, work-rooms for this purpose may be located anywhere. If possible, several rooms should be secured and apportioned to the various kinds of work, e.g., general laboratory rooms, chambers for special workers, sterilization-room, thermostat-room, cold-storage and stock-culture rooms, storage rooms for chemicals, small

glass-inclosed rooms for transfer of cultures, photographic rooms, dark rooms for developing, etc. The general photographic rooms should have overhead light as well as side light.

## EQUIPMENT OF THE LABORATORY.

Many pieces of apparatus may be procured from time to time, as the exigencies of the work demand or as the funds will permit. Other apparatus must be provided on the start, and some of it when the building is constructed or reconstructed.

\*FIG. 79.—Small portion of a cabbage leaf from Long Island, New York, showing characteristic water-pore infections due to *Bacterium campestre*. The blackened veins correspond to the location of the bulk of the bacteria which have gained entrance to the vascular system of the leaf by way of the groups of water-pores situated on the serratures of the leaf, particularly those which are conspicuously blackened. Those parts of the leaf where only the larger veins are shown were green and normal in appearance. Coll. July 16, 1902. Drawn from a photograph.



Engine-room, showing steam-pump for exhaust-air and compressed-air work.  
Engine built to order by Gould & Garrison, Brooklyn, New York.



There should be hot-water pipes, cold-water pipes, steam pipes, a steam bath, gas-pipes, compressed-air pipes, exhaust-air pipes (plate 10 and fig. 81), and electrical wires for light and motive force. There should be thermostats, water-baths, cooled rooms, ice-boxes, steamers, dry-ovens, autoclaves, a distilled-water outfit, an alcohol-still (by which waste alcohol may be recovered or absolute alcohol prepared), an ether-still, filters, gas-generators, gas-furnaces, anaerobic apparatus, the very best microscopic outfits including apochromatic lenses, photographic and photomicrographic appliances, liquid-air receptacles, cylinders of compressed carbon dioxide and oxygen, microtomes, paraffin baths, glassware, balances, chemicals, and many minor pieces of apparatus.

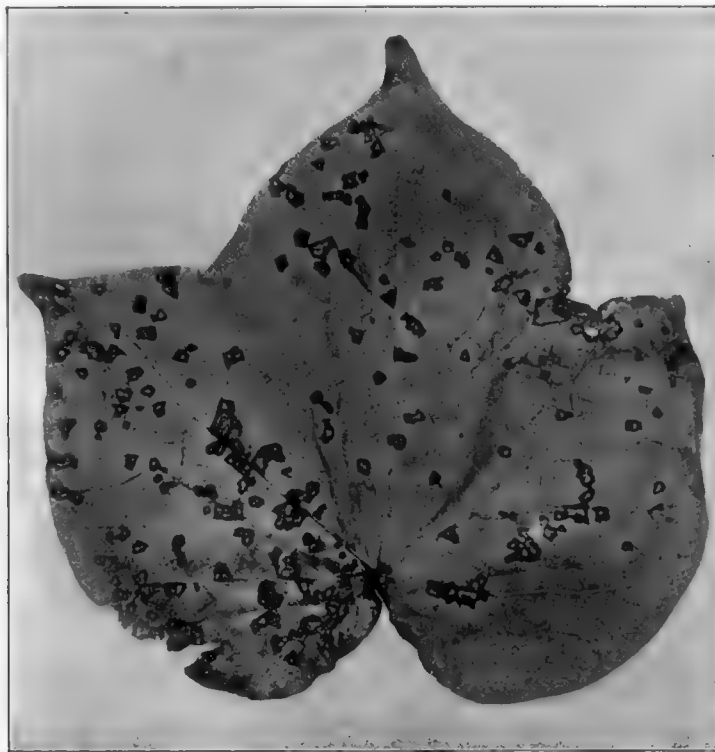


Fig. 80.\*

\*FIG. 80.—Angular leaf-spot of cotton in which stomatal infections appear to be the rule. This leaf represents the secondary stage of a natural infection, *i. e.*, the spots have browned and shriveled, and they involve the entire thickness of the leaf. In an earlier stage of the disease the spots are limited to the under side of the leaf (mesophyll), and occur in the form of small water-soaked, uncollapsed areas surrounding stomata, under which nests of bacteria occur. These spots gradually deepen so as to involve the palisade tissue, and then they become visible on the upper surface of the leaf. The spots are not yet shriveled or browned, but if the leaf is held up and viewed by transmitted light they appear as translucent areas, while by reflected light they are dull and wet-looking. A little later they present the appearance shown in this figure. The writer has obtained all stages of this disease in Washington by spraying upon the plants young agar cultures of *Bacterium malvacearum* suspended in sterile water.

In general, the working capacity of a laboratory will be greatly increased by giving the director a stipulated sum of money per annum and carte blanche to buy laboratory necessities whenever and wherever and in whatever quantity he sees fit, requiring only that he submit vouchers; also by the employment of a number of subordinate assistants of special fitness, to whom may be assigned much of the purely mechanical and routine work of the laboratory, such as the proper cleaning of glassware, the making of ordinary culture media, the keeping alive of stock cultures, the preparation of staining media, the embedding, cutting, and staining of microtome sections, the making of photographs, the indexing of literature, etc.

No scientific man should be willing to trust any piece of work in his own line to an assistant unless he can do it as well himself, or better, but when it has become to him the merest routine, his time, if worth anything, can be more profitably employed in something else. In most American laboratories which the writer has visited there is a woeful lack of intelligent subordinate assistance, such, for example, as that furnished by the German "Diener" and the Malays of Java. Every assistant can not hope to become at once an independent investigator, although, if competent, his work should always be shaped toward this desirable end.

A good library should be within easy reach, and as a suggestion to this end a list of useful books and papers is appended under the head of Bibliography of General Literature. A card catalogue of current literature is also very useful and in time becomes invaluable if properly made.

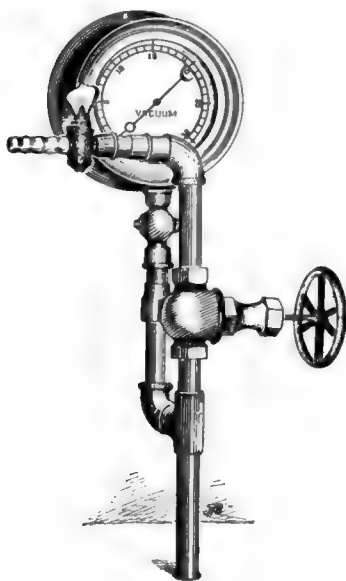


Fig. 81.\*

#### CARE OF THE LABORATORY.

The laboratory should be a clean place. Its walls should be of such material that they can be rinsed or wiped down occasionally. The floors, doors, tables, window-sashes, etc., should be wiped every day, every other day, or at least every third day, with clean cloths wet in distilled water, boiled water, or clean lake or artesian water. The use of river water, swarming as it does very frequently with all sorts of bacteria, is not to be commended for cleaning purposes, and brooms should be taboo. No one should enter the laboratory who has not business there, and order and quiet should prevail.

\*FIG. 81.—End of the vacuum-pipe on laboratory table. The gage serves to show the degree of exhaustion, *i. e.*, whether there is any leak in the piping between the engine-room and the laboratory. The two rooms should be connected by a speaking-tube.



## PREPARATION AND CARE OF CULTURE MEDIA.

Everything should be carefully weighed or measured. Everything should be clean as possible to begin with. By water is usually meant distilled water, and this should be free from copper or other germicidal metals (see Bolton, Bibliog., XXXVIII). Moore & Kellerman have shown very recently that the *Bacillus typhosus* is destroyed in distilled water if the merest trace of metallic copper is present. Water swarming with this organism was sterilized simply by standing three hours in a copper vessel. The writer found the count of *Bacillus tracheiphilus* reduced over 30 per cent by exposure in bouillon in block-tin tubes for twenty-one hours. Exposure for forty-eight hours gave the same result, *i. e.*, 33 per cent destroyed. A simple glass still is shown in fig. 82. As far as possible the chemicals should be

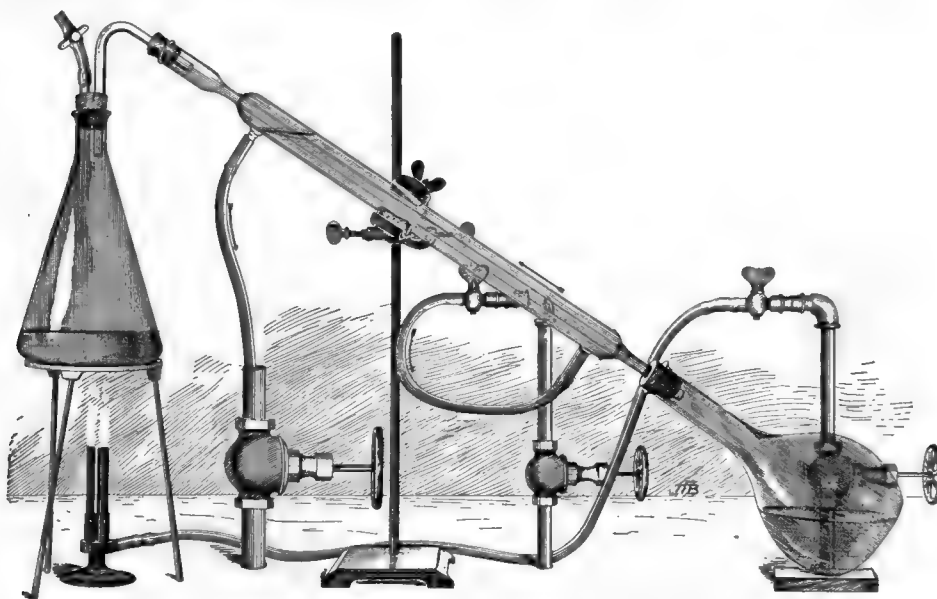


Fig. 82.\*

c. p., and in many cases it is necessary to make the test for oneself, no matter what the reputation of the firm or the statement on the label. When possible, broken packages should be avoided. It is therefore best to procure most chemicals in several small packages rather than in one large one. If the preparation of culture media is broken off before its completion, by nightfall or interruptions of any kind, the unsterilized or incompletely sterilized media should be put into the ice-box, especially if it is warm weather. Neglect of this precaution frequently results in the spoiling of the media. In steam sterilization one should begin to count time only after the thermometer registers  $100^{\circ}\text{C}$ ., or at least  $99^{\circ}\text{C}$ . Those who live in high

\*FIG. 82.—Portion of a work-table showing method of distilling water for use in making culture media. The flasks should be insoluble glass. The cold hydrant water passes through the condenser in the direction of the arrow. In actual use the upright flask and the flame are sheltered from air-drafts by sheet asbestos. One-ninth actual size.

mountain regions must use autoclaves. Agar, potato, etc., in test-tubes, may be steamed twenty minutes on each of three consecutive days. Gelatin, beef-bouillon, and all other fluids likely to be injured by long heating should be steamed only ten or fifteen minutes on each of three consecutive days, if in tubes. The writer frequently steams such media fifteen minutes the first day, ten minutes the second, and five minutes the third. Agar, gelatin, bouillon, etc., stored in flasks in large quantity must be steamed a longer time—usually thirty to forty-five minutes on each day.

The first steaming, when softened gelatin is added to bouillon, usually requires thirty minutes. To melt flaked agar quickly, shake it into fragments or break it with a sterile glass rod before putting it into the steamer.

Oversteaming should be carefully avoided. It softens gelatins or altogether prevents their solidification, and is very apt to cause troublesome precipitates in a variety of media. Precipitates in bouillon often occur if the tubes are not clean, or if the bouillon was not well boiled at first before filtering and placing in tubes. If the beef-broth looks greenish in the beaker or flask, rather than a clear yellow, it may be assumed that it needs more boiling and that if tubed in this condition it will throw down whitish particles on subsequent steaming. The writer prefers to obtain his ordinary + bouillons by incomplete neutralization with sodium hydrate rather than by addition of hydrochloric acid after full neutralization. The adding of hydrochloric acid precipitates out certain nutrient substances and also seems to interfere with the growth of some organisms. Distilled water and river water should be sterilized in quantity in the autoclave. For details concerning the making of particular media the student

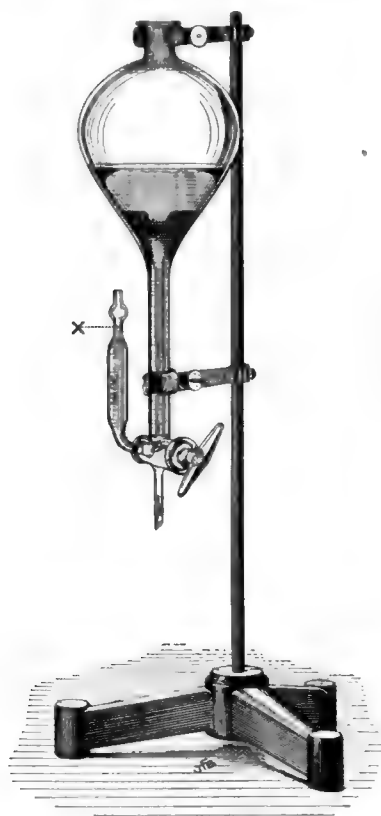


Fig. 83.\*

should consult the standard text-books, a dozen or more of which should be kept within easy reach in every laboratory. Some formulæ are given in the middle part of this volume. The autoclave may be used for the preparation of sterile water and some media, but, in general, I prefer media which has not been heated above  $100^{\circ}$  C., especially for use with sensitive organisms. Media should be heated in the autoclave only for a brief time and at a minimum pressure, generally not more than ten minutes and at not more than  $110^{\circ}$  C. Milk, gelatin, and media containing sugars should never be sterilized in the autoclave. Sugars

\*FIG. 83.—Apparatus for rapidly filling test-tubes with 10 cc. portions of agar, bouillon, etc. By means of this device an expert assistant can fill 500 tubes an hour. Made to order by Emil Greiner. Height, 23 inches. The bulb above X is essential.

and other substances decompose at these high temperatures and the results obtained by the growth of bacteria in such media are not comparable with those obtained on media sterilized at 100° C. Hitchens has recently shown that detrimental acids are formed when bouillon containing sugar is autoclaved. Peptone water, agar, and bouillon may be sterilized in the autoclave. For titrating culture media the writer uses the burettes shown in fig. 59. The twentieth-normal alkali is stored as shown in fig. 60. Quadruple-normal sodium hydrate solution is used for neutralization. The phenolphthalein solution is made by adding 1 gram of the dry powder to 100 cc. of 50 per cent alcohol, and then enough  $\frac{N}{20}$  sodium hydrate to carry it fully into solution, removing the yellow color without making the fluid

a very decided pink. Fluid media may be filled into tubes very rapidly by means of the device shown in fig. 83. For storing media sterilized in test-tubes and for holding cultures made on such media the writer has found ordinary quinine cans very useful (fig. 84).

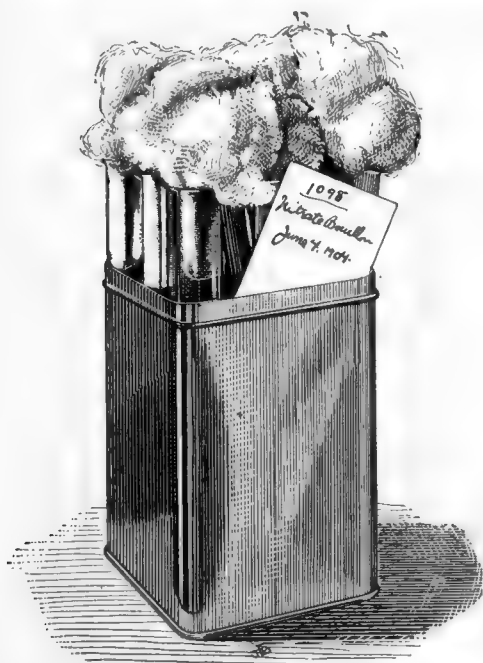


Fig. 84.\*

plug, dipping the lower end of it quickly into and out of hot sterile paraffin, and replacing it in the mouth of the tube or flask before the melted paraffin has had time to cool, answer the purpose very well, but have the objection that all of the tubes must be placed in turpentine or some other solvent of paraffin before they can be cleaned for a second use. On the whole, the use of moderately tight plugs and the storage of the media in cool or cold air are the best methods of retaining the water content of the medium. Nutrient media should be made in small quantities and often, rather than in large quantities and at infrequent intervals. The cotton should be dry-heated in bulk before plugs are made from it.

\*FIG. 84.—Ordinary quinine cans with a little cotton in the bottom are very convenient for holding cultures and culture-media in test-tubes. One-third actual size.

## THE CLEANING AND STERILIZATION OF GLASSWARE AND INSTRUMENTS.

New glassware may be boiled in soap-suds, rinsed thoroughly, soaked in the chromic-acid cleaning mixture for some hours, rinsed in hydrant water, soaked in several changes of distilled water, soaked or shaken in alcohol, and finally rinsed in distilled water. Neglect to wash in alcohol will frequently leave behind on the walls of the test-tubes an invisible film which causes vexatious precipitates in beef-bouillon, etc. Discarded tubes, flasks, and dishes containing living organisms must be autoclaved or filled with the chromic-acid cleaning mixture before they are washed. Some responsible person should attend to this. If acid is used it should be allowed to act for some hours.

Petri dishes should fit together well, but not tightly, and should be double-wrapped in clean Manila paper before placing them in the hot-air oven, or else should be inclosed in suitable tin boxes. The writer prefers to wrap them. The paper for this purpose may be 12 by 12 inches. The dish should be placed in the

middle. The sides of the paper are folded over it; the corners of the projecting ends are then turned in, leaving V-shaped flaps, which are folded down on to the plate. The second covering is folded at right angles to the first and on the other side of the dish. Dishes treated in this way and ready for sterilization are shown in fig. 85. Pipettes should be dry-heated in the tin boxes already mentioned (fig. 37) after having the upper end carefully plugged with cotton, which should not



Fig. 85.\*

project. Knives, scalpels, scrapers, spatulas, needles, forceps, etc., may be sterilized in the Bunsen flame, or, if needed cold in quantity, may be wrapped in Manila paper or put uncovered into short tin boxes and heated in the dry oven at 140° C. for two hours. Petri dishes, test-tubes, and all other apparatus wrapped in paper and put into the oven for sterilization by dry heat should have air spaces between them, *i. e.*, they should not be crowded together tightly, and the recording thermometer should project well down into their midst. The investigator should test the behavior of his oven when full and empty. Many cheap ovens give very different temperatures in different parts, especially if filled with apparatus, so that cotton or paper may be scorched in one part and not sterilized in another. The best oven known to the writer is that made by Lautenschläger. The improved form of the Lautenschläger oven shown in plate 6 does not require watching and gives a uniform temperature

\*FIG. 85.—Petri dishes wrapped in two layers of Manila paper and ready to be dry sterilized. They are set on edge in the oven.

in all parts. It also furnishes a maximum temperature with a minimum consumption of gas, hot air being fed to the flame. The apparatus has an inner, outer, and middle wall. A horizontal iron gas pipe, of the relative size shown in the front of the picture, passes entirely around the apparatus at the bottom between the outer and middle wall. On top in this tube are many small openings through which gas escapes and when lighted forms so many small Bunsen flames. Air is drawn

in at first and mixed with the gas in the middle open part of the feed pipe in front. The products of combustion escape through the chimney on top of the oven. There are pilot lights, so that the apparatus is set going easily. The result of this arrangement is that the middle wall becomes heated very hot, and consequently the air between this wall and the inner wall rises, cool air entering through holes in the bottom to take its place. There is thus created a powerful upward mount of hot air. This enters the oven through several hundred holes in its ceiling, is forced downward and escapes through as many holes in the floor. From this place the hot air is continually crowded sidewise and

backward through brass tubes into the furnace chamber where it serves to support the combustion.

Unless the dry-oven has a very uniform temperature throughout, so that there is no danger of scorching the cotton, plugged test-tubes should be tied together loosely and stood on end, cotton uppermost. Petri dishes (wrapped in paper as directed) may be set on edge. If the test-tubes have been properly cleaned, dry-heating is not necessary for such as are to hold steam-heated media, provided the cotton used for the plugs is dry-sterilized in advance. The best surgeon's absorbent cotton

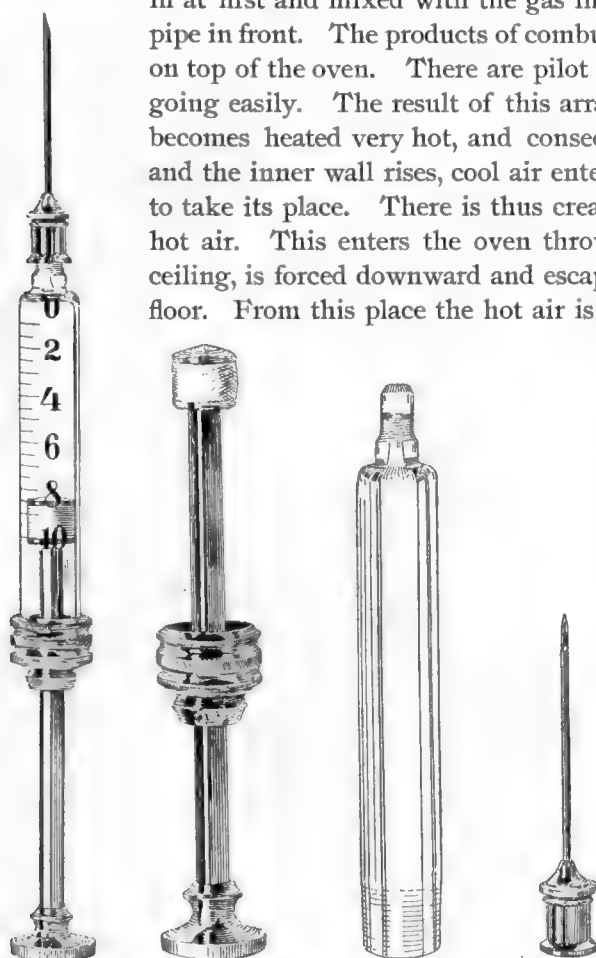


Fig. 86.\*

is not too good for this work. It should be unrolled and put into the dry-oven in a loose armful and heated just below the scorching point for several hours (2 to 3 hours at 145° C. will answer), with occasional unfoldings and turnings so that all parts may be heated uniformly. It is now taken out, re-rolled and put away in clean paper until needed. By this means all fungous spores lodged in it are destroyed and

\*FIG. 86.—Dr. George Meyer's hypodermic syringe, made by Lautenschläger. Desirable on account of perfect workmanship, and because it is easily sterilized without injury. This size holds 1 cc. By twisting the button of the piston the packing at the other end is tightened or loosened at will. The separate parts are enlarged one-fourth.

an oil is driven off which otherwise would be deposited as a whitish distillate on the inside of the test-tubes near the plugs. Hypodermic syringes may be sterilized by boiling in distilled water if the contaminating organism is non-sporiferous, or by soaking twenty-four hours in 5 per cent carbolic-acid water or lysol water and

a subsequent soaking and boiling in pure water. The writer prefers the Meyer syringe, made by Lautenschläger (fig. 86). Syringes which allow the culture media to ooze out around the piston whenever any strong pressure is exerted are dangerous and should never be used with infectious material. Those which do not admit light or allow the experimenter to see how much fluid has been used or whether air is present are unsatisfactory. In case of many plants, needle-pricks are more satisfactory than hypodermic injections (pl. 4 and figs. 8 and 88). Needles are sterilized in the open flame as needed.

When conveniences are not at hand, as on long trips in the country, the kitchen-oven may be used for sterilizing glassware, or even an open flame (alcohol lamp), and agar and gelatin for the making of poured plates may be melted by placing the tubes in hot water in a tin cup or tea kettle, but, in general, the writer has not found the rooms of ordinary farm houses very well suited for research work. Usually they are too dusty.

Surgeon's gauze is very convenient for laboratory use, for coarse filters, wipe-cloths, etc.

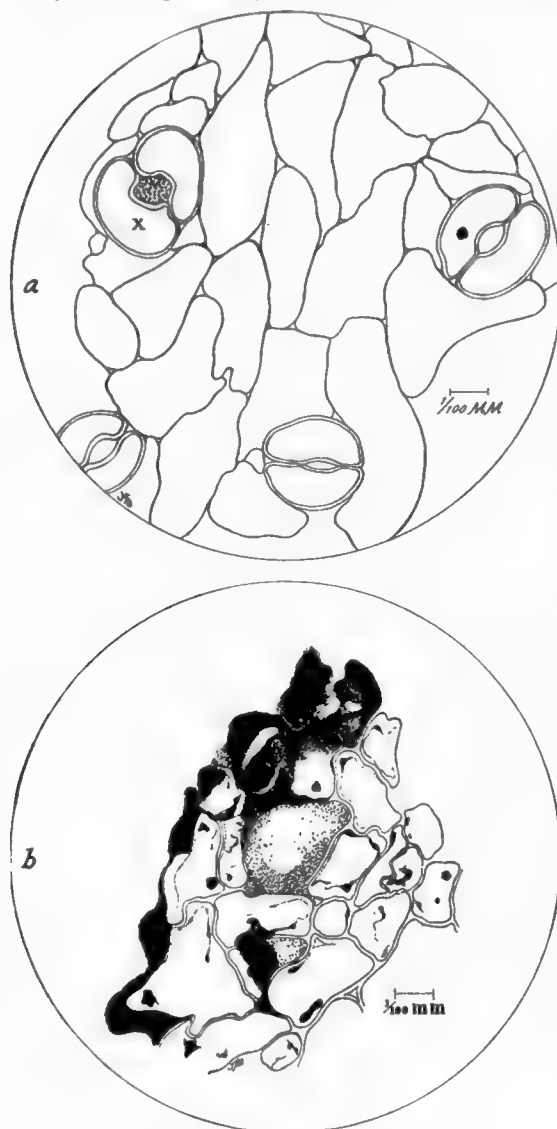


Fig. 87.\*

\*FIG. 87.—Early stage in the infection of a cabbage leaf by *Bacterium campestre*; a, epidermal layer on the apical part of the tooth of a leaf, showing one of the four stomata (X) full of bacteria. For the condition immediately under X see b, which was drawn from the third section in series, the intermediate one including part of the guard-cells. Slide 338, B1, stained with carbol-fuchsin. Drawn with the Abbe camera, 3 mm. Zeiss apochromatic objective and 12 compensating ocular. Material collected and fixed 8 days after infection, which was accomplished by atomizing upon the plant water containing a pure culture of *Bacterium campestre* grown on slant agar. When collected many of the serratures had begun to show traces of the brown stain which invariably appears when this organism grows in cabbage. The plant was inclosed in the cage shown in fig. 95, and was extruding fluid from its water-pores when it was sprayed.  $\times 500$ .

## THE MAKING AND TRANSFERENCE OF PURE CULTURES.

In addition to what has been said under *Pathogenesis*, the following suggestions may be of service to the beginner.

For the making of plate cultures and for the transfer of organisms from one culture medium to another, select a still day and, if possible, a day when a gentle rain or snow is falling. This offers ideal conditions, since the earth is wet, the outside air has been washed free from dust, and there is no wind to stir up dust within the laboratory. A strict adherence to this rule is sometimes very inconvenient and it is



Fig. 88.\*

not meant to be iron-clad. It is, however, of immense service in keeping cultures free from contaminations, and those who propose to disregard it should remember that haste in the beginning of an experiment often leads to vexation and delay in the end, especially when the success of the experiment depends absolutely upon the purity of the culture.

\*FIG. 88.—Soft rot of green cucumbers inoculated by needle-punctures from a pure culture of *Bacillus carotovorus*. The only parts not softened are those through which the infected needle entered, i. e., the parts rubbed with mercuric-chloride water. In each a little button of tissue under the disinfected area did not decay. The sound fruit at the right was punctured at the same time, but with a sterile needle. The cucumbers had been removed from the vine, but were not flabby. They were exposed after inoculation to the ordinary air of the laboratory. The photograph was made on the seventh day. About two-fifths natural size.



When ready to make the transfers or to pour the plates, close the windows, wipe up the tables, and wet down the floor, window-sashes, etc., with distilled water or boiled water, and reduce the air-currents within the laboratory to a minimum (especially when transfers are to be made in the open room) by keeping the doors shut and restricting the movements of all persons who may be in the room. It is much better to do all of this work in specially constructed small rooms (plate 11) than under hoods (plate 12). Hoods are open only in front. They may be made of any convenient size. The one here figured is 32 by 39 by 20½ inches, outside measurements. When one is far from laboratories small hoods may be extemporized out of clean paper, or cultures may be poured and transfers made inside of a clean pail or jar, turned down on its side. Any method, in fact, which restricts the movement of air past open plates and tubes will be found serviceable.

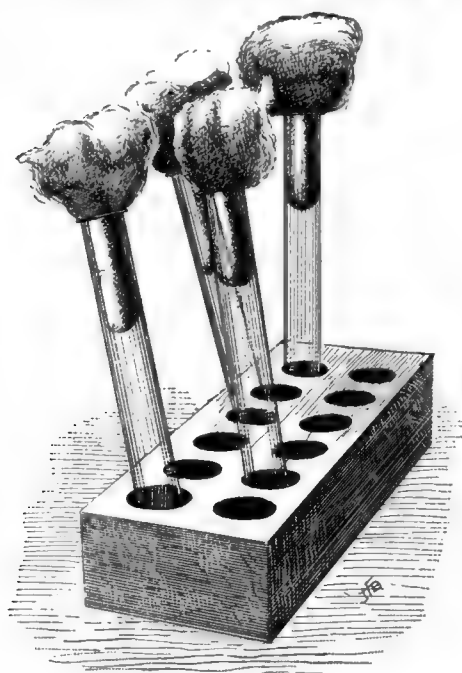


Fig. 89.\*

The work-shelf of the room shown in plate 11 faces a window as wide as the room, and extending from the level of the shelf to the height of the other windows in the room. This window faces south and is only 6 feet from a well-lighted window in the outer wall of the building. The room also receives bright light from the west side. At the front end of the shelf are a Bunsen burner with cut-off flame, a box of safety matches, a box of rubber bands, and two tumblers—one for burned matches and one for platinum loops, needles, forceps, etc. Immediately under this part is a narrow drawer for pencils, note paper, knives, etc. At the back end are a few wrapped Petri dishes, a nivellation apparatus, a flask of sterile water, and a crate of media. Underneath this part is

a second shelf 3 inches below the first, where Petri dishes and tubes containing solid media may be put out of the light as fast as inoculated. The size of this room (inside measurement) is 4 by 4 by 10 feet, and it is large enough. No provision is made for ventilation, because air-currents in a culture-room are very objectionable. The windows, walls, and floor are wiped up with distilled water before making transfers. Outside is a bit of the author's private laboratory. At the right is the microtome and behind it on the wall are deep and shallow drawers; 69 is for bulk paraffin; 70 A, B, C, D, E, are cut into small compartments used for paraffin blocks. The very shallow drawers are for ribbons which can not be mounted the day they are cut; 72 has a series of shelves opening on the south side and is used to hold photographic printing frames.

\*FIG. 89.—Pine block with inch holes, convenient for holding test-tube cultures during examination, or tubes of media which are to be inoculated. A good size is 9½ by 3½ by 1¾ inches.





The author's culture-room.

At the left hand (back) are narrow shelves for culture-media, pipette-boxes, etc. At the right is the work-shelf, covered with plate glass.



The agar may be poured at 42° C. in case of organisms whose thermal death-point is known to be high (50° C. or above). For all others it must be cooled carefully to 40° C. before inoculating for poured plates. This requires five or six minutes in the water bath at 40° C. Even this temperature is too high for some organisms and then gelatin at 30° C. may be used. When ready to pour, take a clean absorbent cloth and carefully wipe all water from the outside of the tube (the lips of which have been previously flamed *gently* with a rotation of the tube on its long axis), lift the cover of the dish only as much as is necessary, hold the cover *over* the dish (not at one side), pour quickly but gently, and re-cover, tilting the dish about quickly but gently, if the fluid has not already covered the bottom. To entirely cover the bottom sometimes requires a smart little jerk, if the agar is not very fluid. The student must learn to work rapidly and dextrously, then there will be no complaint that the agar has solidified before the plates are poured. The

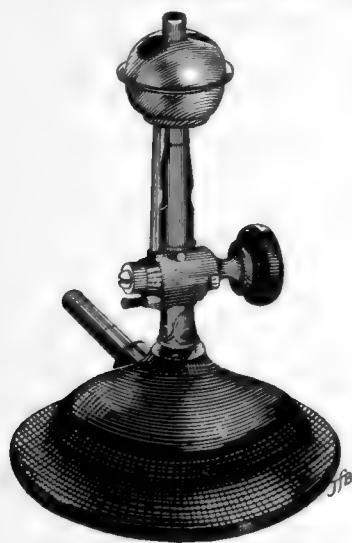


Fig. 90.\*

plates should be set on a level shelf while the agar or gelatin is hardening, or, if the colonies per square centimeter are to be determined, a nivelling apparatus such as that shown in fig. 66 must be used, and the dishes should have flat bottoms. When plates have been inoculated too abundantly to secure subcultures from single colonies, these may sometimes be obtained from the traces of agar or gelatin left in the tubes from which the plates were poured. With this end in view, these tubes should be re-plugged and laid away, for a few days, the lips and top of the tube which were wet by the agar or gelatin being first heated hot in the flame, care being exercised not to crack the tubes.

All tubes containing fluids should be opened and inoculated in a position as nearly horizontal as their contents will permit, and tubes of solid media, such as agar, may be held level or inverted for inoculation. A convenient block for holding test-tube cultures during examination is shown in fig. 89. It is usually best to flame the plugs slightly before their removal, particularly if they have been exposed to the air for some days. As an additional precaution the transfers should be made under a glass hood, or in a special culture-chamber. If sterilized needles, loops, knives, forceps, pipettes, or anything else designed to be used in making the transfers have accidentally touched *anything whatsoever*, they are presumably contaminated and must be rejected or reflamed. Do not handle the lips of test-tubes containing gelatin or agar from which plates are to be poured. Your hands may be contaminated by resistant spores. Take hold of the tubes lower down. To economize gas and avoid heating the air of the small work-chamber to an uncomfortable degree, small, cut-off, constant-flame burners are very convenient (fig. 90).

\*FIG. 90.—A constant Bunsen burner with cut-off flame. Very useful for the laboratory table and the culture room. About two-fifths actual size.

Plates, tubes, and flasks containing pure cultures or designed for inoculation should *never* be opened in the general laboratory on a windy day or in air currents. Pour two uninoculated agar or gelatin plates in the proper way. Keep one covered and uncover the other for a few moments in a current of air, *i. e.*, as long as the time required to make a plate culture. Then keep the two plates together and compare from time to time. A few experiments of this sort will convince the most skeptical of the necessity of avoiding drafts.

The person and clothing of the experimenter should be as clean and free from dust as possible. White duck coats are very convenient. They show at once when they are soiled and need washing and ironing.

Organisms which for some reason may be difficult to obtain in ordinary plate cultures and which differ markedly from their associates in some particular way, *e. g.*, by more rapid growth, by indifference to heat, to acids, to thymol, to chloroform, to absence of air, etc., or which can use, as food, substances which will not support the growth of most bacteria, may sometimes be isolated very readily by providing conditions suited to their growth and unsuited to that of the bacteria with which they are mixed. This is Winogradsky's principle of *elective culture*. As he defines it, this is a culture "which presents conditions favorable only to a single definite function or, more exactly, to a function as strictly limited as possible." Such



Fig. 91.\*

media or methods are exactly the opposite of universal. Nutrient starch jelly and nutrient silica jelly are good examples of such media. Nutrient fluids rich in acid potassium phosphate or destitute of nitrogen are additional examples.

Heat is often an excellent means of separation. Winogradsky separated his *Clostridium pasteurianum* from all but two of the contaminating species by heating ten minutes at 75° C. (Archives des Sci. Biol., Vol. III, p. 310). The isolation of *Streptococcus (Leuconostoc) mesenteriioides* by Liesenberg & Zopf and of *Bacillus hortulanus* by Sturgis are other examples of separation by heat. Omélianski's separation of his hydrogen-cellulose ferment from his methane-cellulose ferment by exposure of the recently established methane ferment to 75° C. for fifteen minutes is another good example.

#### THE FINAL DISPOSAL OF INFECTIOUS MATERIAL.

Diseased material should not be left around the laboratory any longer than is necessary. When it has served its immediate purpose that which is not to be preserved permanently should be thrown into the furnace. Small amounts may be sterilized by putting into beakers or jars and covering with cleaning mixture or equal parts of crude sulphuric acid and water. Crude vegetable and animal sub-

\*FIG. 91.—Instrument for making puncture-inoculations. It consists of a bone handle with a metal-screw socket, into which a sewing needle is thrust. The needle is usually of small size—a No. 8 or 10.



Work-table with movable frame of wood and glass.

Bacteriological transfers may be made under this frame in the open room if windows and doors are kept closed.



stances likely to become moldy must never be stored in refrigerators designed for pure cultures. The open ice-box is the proper place for such substances, and they must not be left there indefinitely. Some people have a mania for collecting everything and then keeping it a long time without making any use of it. An ice-box treated in this way soon becomes an intolerable nuisance.

Discarded plates, tubes, slides, covers, pipettes, contaminated litmus paper, etc., should be autoclaved, or covered or filled with cleaning mixture, or dropped into it, as the case may be. Deep, narrow glass jars or long, rectangular enameled pans are necessary for the pipettes. Soiled hands may be disinfected with mercuric-chloride water (1:1000), which should always be on hand in the laboratory in quantity properly

labeled. Slight wounds should be washed five or ten minutes in this fluid. Surfaces of floors, tables, etc. soiled by spilled bacterial cultures should be covered immediately with mercuric-chloride water (1:1000) and wiped up carefully after ten or fifteen minutes with distilled water. Spilled cultures of molds should be soaked in mercuric chloride (1:1000) for at least an hour before wiping up. Neglect of these simple rules means the seeding down of the ice-boxes, culture-chambers, and the general laboratory with all sorts of resistant mold spores and bacteria. An abundance of cheap carbonate of lime should be kept on hand for the prompt neutralization of spilled acids. A mass of cotton waste is convenient for the prompt mopping up of spilled fluids.

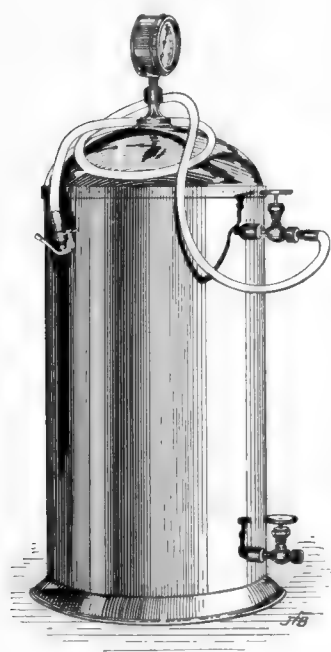


Fig. 92.\*

All contaminated needles, loops, knives, scissors, forceps, etc., may be sterilized in the open flame. Instruments which are too valuable to be flamed may be sterilized in carbolic acid (5 per cent) or formaldehyd (5 per cent) or lysol (5 per cent). *Never* put down a platinum needle or loop which has been used

in making transfers until it has been passed carefully *its whole length* through the flame. Dissections are best made on trays which can be easily cleaned and sterilized.

\*FIG. 92.—Compressed-air tank and spray-tube. The one here shown, made by Boeckel, Philadelphia, is nickel-plated and very substantially constructed. It is filled by means of a small pump similar to a bicycle pump. The gage registers up to 100 pounds per square inch, but 40 pounds pressure is ample. The bacterial fluid is placed in atomizers of the form shown in fig. 93. The method of attachment is not satisfactory. This device is very convenient when trees or low plants covering a considerable area are to be inoculated. Height, 29 inches. The same firm has devised a compact traveling outfit, the compressed-air tank being about one-half the size of the one here figured. The whole is packed into a neat portable box, and the only disadvantage is the small size of the air-chamber, which requires more frequent pumpings. Of course the apparatus may be used equally well for the distribution of fluid germicides or insecticides.

## METHODS OF INOCULATION.

Inoculations may be by punctures with a delicate needle (fig. 91), by abrasions of the surface, by hypodermic injection, by watering the soil with infective material, by plunging aerial parts into infectious liquids for a longer or shorter time, by simply putting the bacteria into drops of water on parts of the plant and protecting from sunlight and evaporation for some hours, or on a larger scale by spraying portions of the surface with very dilute culture fluids or, preferably, with water containing the bacteria (figs. 92, 93, 94), by brushing or rubbing cultures into some part of the surface, by allowing insects, snails, etc., to feed on diseased material and then colonizing them on healthy plants. The writer has made good use of this last method in case of three different bacterial diseases. Stomatal infections may be secured by subjecting the plants to conditions similar to those occurring in nature on dewy nights or during heavy fogs or prolonged rains, *i. e.*, by placing the potted plants on wet sand, atomizing thoroughly with sterile water and covering with tall, roomy bell-jars. The experiment should be undertaken in a cool rather than a warm house. When

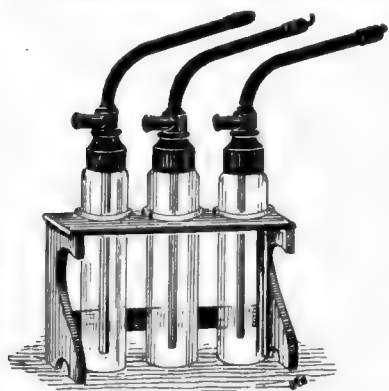


Fig. 93.\*

the right conditions have been obtained, moisture covers the surface of the plant in tiny drops which do not evaporate. The bell-jar may now be raised and the plant again atomized lightly with sterilized water containing the bacterium. The best time to do this is late in the afternoon, so as to take advantage of the cooler night temperature. When the bell-jar is returned, which should be immediately after spraying, it should be covered with cloth or paper to protect from the light. Usually bell-jars should be removed at the end of twenty-four hours, but exceptionally they may be left on thirty-six to forty-eight hours, if not

exposed to the sun. Inoculation cages are very convenient for small plants (fig. 95). In case of trees, or shrubs, or masses of tall herbs, tight-fitting covers of tent-cloth will be found serviceable for obtaining conditions similar to those prevailing in wet weather. They may be left on 1 to 3 days, the outside of the tent as well as the plants within being sprayed with water often enough to keep everything moist until infections have been secured.

When the nature of the plant will permit it and when only a few inoculations are to be made, the surface which is to be punctured should be rubbed thoroughly for three to five minutes with mercuric-chloride water (1:1000) and then

\*FIG. 93.—Atomizers for use with the air-tank (fig. 92). These are made by the Davidson Rubber Company, Boston, Mass. About one-fourth actual size. The De Vilbiss sprayer, made in Toledo, Ohio, and now used by the writer, has several distinct advantages. It is all metal and can be sterilized in boiling water without becoming twisted out of shape, it can be attached more easily to large flasks and to the tube leading from the compressed-air tank, and the spray may be directed up, down, or straight ahead without changing nozzles. It requires, however, more force to operate than the Davidson sprayers, and consequently is less convenient when used with a hand-bulb.



washed with equal care in sterile distilled water. When many inoculations are made with large numbers of check plants and when due care has been taken to work under conditions such that accidental contaminations from the same organisms are not to be feared, the writer has not found this precaution necessary. The use of mercuric chloride should be avoided, if possible, especially on leaves, as the writer's experiments have shown that it penetrates into the plant (some plants) for a considerable distance and prevents the action of the bacteria to this extent (fig. 88), if not altogether, as has happened in some cases.

#### THE KEEPING OF RECORDS.

If one contemplates doing much work, a careful record of what has been done is as important as the experiment itself, since exact remembrance is certain to pass away with lapse of time.

In all his work, the student should accustom himself to make very exact statements, so that others may be able to follow him. For example, he should not describe his organism as "yellow" or "red" without qualifications, since there are many yellows and reds, but should carefully compare it with some standard color-scale (Ridgway's, Saccardo's, Standard Dictionary, etc.), and govern himself

accordingly. He should not say, "Organism does not grow at room-temperatures," but rather should state the temperature at which growth does not occur, as  $15^{\circ}$ ,  $25^{\circ}$ , or  $35^{\circ}$  C., any one of which may be "room-temperature," depending on the latitude, altitude, and time of year. He should not say, "Organism is killed at temperature of  $65^{\circ}$  C.," without at the same time stating the age of the culture, condi-

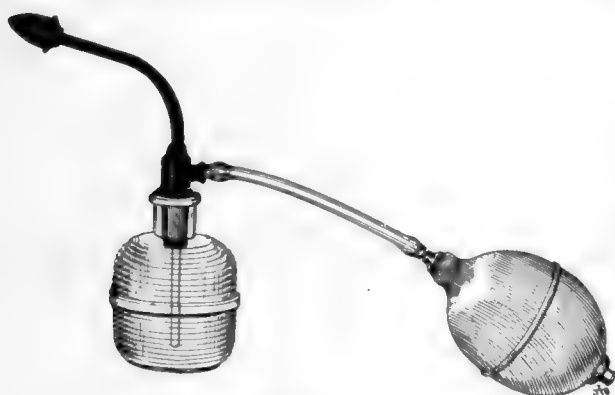


Fig. 94.\*

tions of exposure, and time required, which might be ten days or five minutes.

Every independent worker will in the end devise a method of note-taking which is more or less characteristic of his personal peculiarities and best adapted to his own particular needs. For all persons there is no *one best* method. The methods described in the following paragraphs have been settled upon as those most convenient for the writer, but it does not follow that they are the most economical of time, or the best devisable, or the ones which independent workers should follow. They are here given as hints for beginners and because the method a man employs in his work is always a matter of more or less interest to his fellow-workers.

First of all, there should be provided a record book in which the method of preparation of each culture medium is carefully described. This should be a good-

\*Fig. 94.—Hand-sprayer which may be used for distributing bacteria on plants. Some form is usually kept in every pharmacy and sold as a cologne atomizer.

sized book, well bound in leather, so as to stand long and hard usage. The entire quantity of a culture medium is known as a "stock" and receives a special number, which is written, pasted, or stamped on any flask or tube that contains it and which serves to identify it. If a stock is subsequently divided and a portion of it is treated in some different way, *e. g.*, receives more sugar, acid, or alkali, this portion receives a new number, or the old number with the addition of a letter of the alphabet. Each stock described in the record book is numbered serially from 1, and the book continues in daily use as long as the laboratory, or until it is filled with records and carefully filed away as "Culture Media, Volume I."



Fig. 95.\*

tion. In moist climates, stock quantities of such gummed labels must be kept in air-tight boxes or between sheets of paraffined paper. Test-tubes in crates are kept separate during steaming by writing the number of the stock on a slip of paper and thrusting this into the crate with the test-tubes. The number should be written with a lead pencil. Faber's pencils for writing on glass are useful in case of flasks and

The small pocket ledger, No. 492 of A. C. McClurg & Co., Chicago, is very convenient for certain kinds of notes, especially those made in the field and those required for the identification of alcoholic specimens and stained slides (fig. 112). All records should be in ink, of a sort which does not fade, and in field work a *good* fountain pen is invaluable. Pencil records, especially those made with rapid-writing soft pencils, soon become illegible and should not be tolerated except on paper to be subjected to steam heat.

Large sheets of well-gummed paper should be procured and the labels cut in the laboratory to the size needed. Labels may be cut rapidly in quantity with the apparatus used to trim photographic prints for mounts. When exposed to streaming steam such labels come off easily, and it is best not to paste them on the tubes or flasks until after the final steam sterilization.

\*FIG. 95.—Small cage of wood and glass in which herbaceous plants may be placed for inoculation by spraying. The inside measurements are 12 by 12 by 30 inches. The large door is a great convenience. Hook-fastenings are better than spring catches.

fermentation tubes, since records made with these pencils will bear streaming steam. An inexpensive black pencil which writes on clean glass very readily and bears steam well (even better than Faber's) may be made by stirring into melted beeswax enough lamp-black to make a thick-flowing liquid (as thick as will flow). This is

poured into molds made by wrapping writing paper, in several turns, around a lead pencil or thick glass rod, tying near one end, removing the rod, squeezing the other end flat, turning over its edge, and fastening this flattened end in a split stick or clamp. The paper should be retained as a cover, the string being removed and the loose edge pasted down. A dozen such pencils may be made at a cost of 10 cents. In the absence of such pencils, flasks and fermentation tubes may be distinguished in the steamer by dropping over the neck different-sized rubber bands or different numbers of the same kind of band, or by writing with a lead pencil the number of the stock on a square of letter paper, cutting a hole in its center and slipping this over the neck of the flask or tube. When the steaming is over, the regular labels should be pasted on or the stock number written on with the proper pencil.

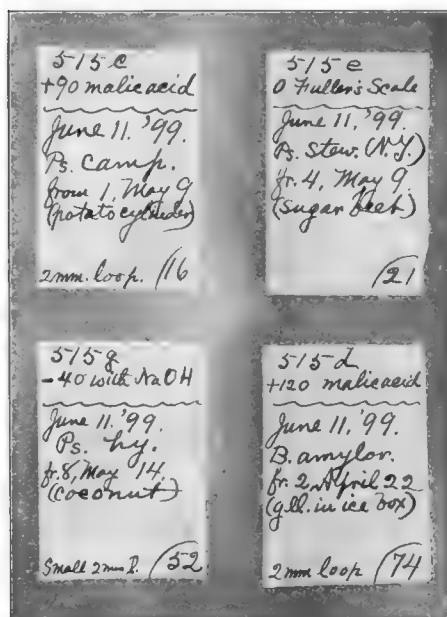


Fig. 96.\*

All plate cultures and all subcultures made on a given day, no matter of what organism, are numbered serially, beginning with 1. These are 1, 2, 3, etc., of that particular day. Those of any other day are also numbered 1, 2, 3, etc. The writer



Fig. 97.\*

usually numbers his plates I, II, III, etc. Labels may be pasted on the covers of the Petri dishes, or all may be done with the glass pencil. Cultures in tubes subject to frequent handling and likely to be needed for some time should have gummed-paper labels written in ink. The above transcripts from labels on four test-tube cultures

\*Fig. 96.—Labels from test-tube cultures.

\*Fig. 97.—Wooden labels from inoculated plants.

(fig. 96) sufficiently indicate what is necessary to form a satisfactory record. This could, of course, be considerably abbreviated by a system of symbols or by depending to a larger extent on the "Notes."

In case of the inoculations, on the contrary, only as many series are made use of as there are diseases under consideration. Each plant is generally given a single number, no matter in how many places it may be inoculated, the separate inoculations being kept distinct, if necessary, by sub-numbers. Each series begins with

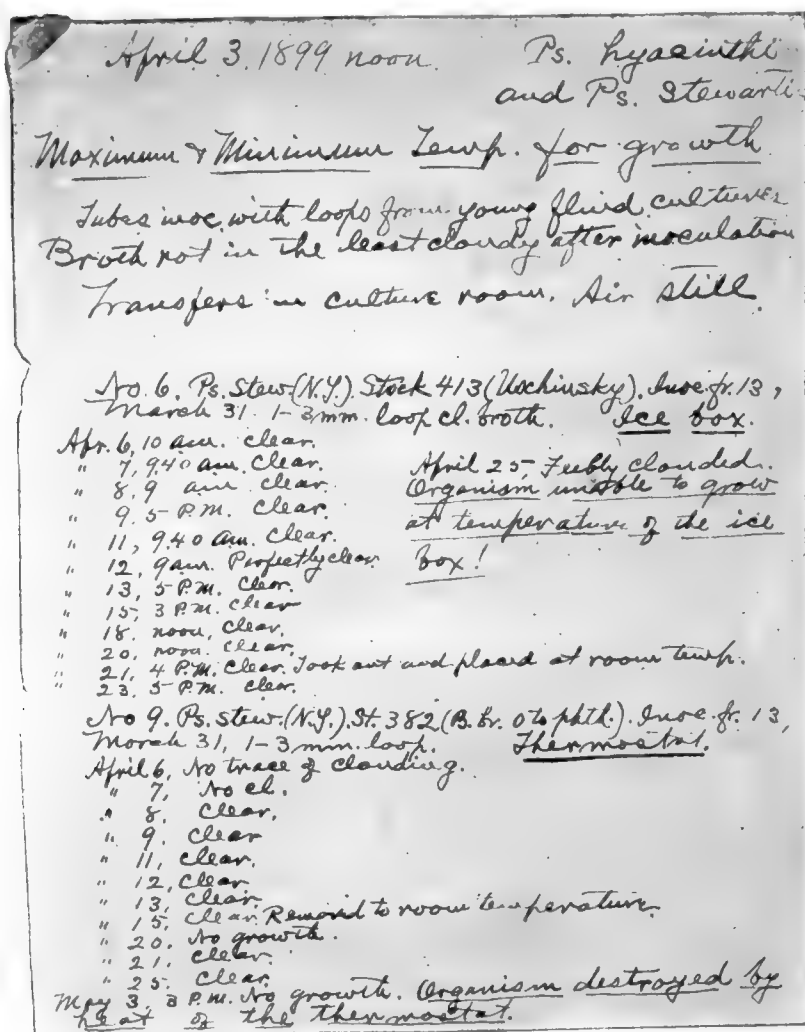


Fig. 98.\*

No. 1 and continues in an unbroken sequence as long as the disease is under consideration. The labels written on soft wood, covered for this purpose on one side with white paint, are stuck into the earth or wired to the plant. Transcripts from two such labels are shown in fig. 97.

\*FIG. 98.—Three sheets showing method of keeping maximum and minimum temperature records. One-half actual size.

After trying various methods, the writer has settled down (in the absence of a stenographer) to the following style of pen and ink notes on cultures, inoculated plants, etc., as extremely flexible and convenient. Reams of ordinary typewriter paper are cut crosswise into three equal portions, so as to form slips about 8 by 3½ inches. As many of these as are necessary for the particular purpose are fastened together at one corner with B, J, N, C, or Z eyelets and the Triumph punch, sold by The W. Schollhorn Company, New Haven, Conn., or by the neat little saw-

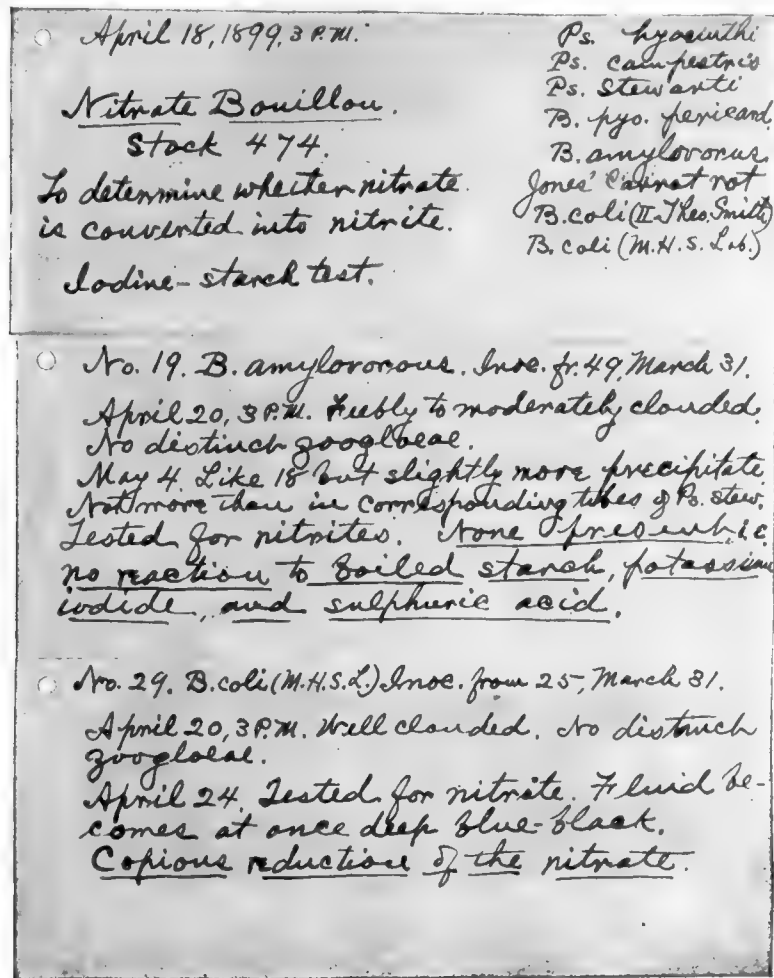


Fig. 99.\*

toothed clamp made by The Middleton P. F. Co., Philadelphia. The first page of the slips is devoted to the name of the organism under examination, the kind of experiment, the date of its beginning, etc. The subsequent sheets are numbered serially and are devoted to particular plants or to particular cultures. If there is an overflow in any particular part of the record, it is very easy to insert additional

\*FIG. 99.—Sheets showing method of keeping nitrate-bouillon records. One-half actual size.

slips. The following transcripts from actual records will serve to illustrate the method (figs. 98 and 99). As fast as the notes are completed they are filed away in boxes or large envelopes until the whole subject has been worked over, when they are sorted out according to their various sub-heads, and all the data which they contain is thus easily available.

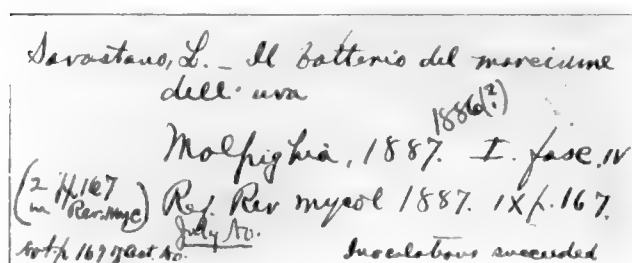


Fig. 100.\*

The writer also uses a stenographer whenever possible, and the typewritten sheets, after immediate careful scrutiny for errors of fact, are filed away in stout Manila envelopes with the name of the parasite written on one corner; 16 by 12 inches is a good size for the envelopes.

Card-catalogues should be made on the L. B. index slips, made and sold by the Library Bureau, Boston, Mass. Figure 100 is a sample from the writer's catalogue by authors. A larger size should be selected if it is desired to include abstracts. When long abstracts or considerable extracts are made from literature which has been borrowed, or may not be readily accessible in future, heavy sheets (6 $\frac{7}{8}$  by 8 $\frac{3}{8}$

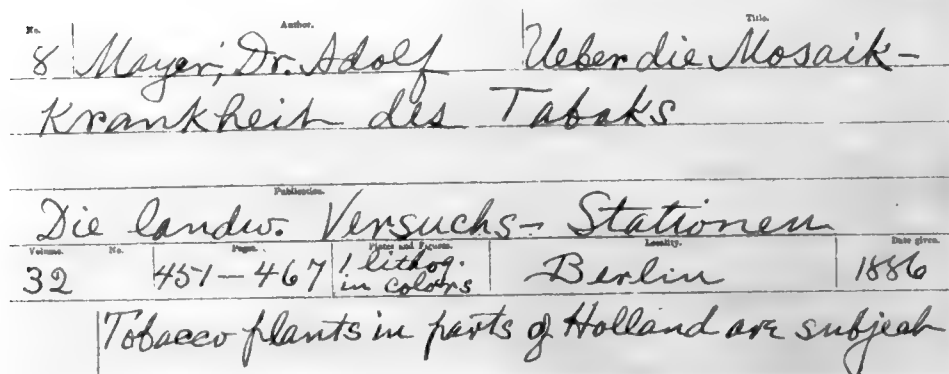


Fig. 101.†

inches) have been used by the writer. These have headlines, as shown in fig. 101, and are preserved by tying into covers made for the purpose. A red line down the left side of the sheet preserves a space for a marginal index.

A serious objection to the making of many abstracts is the time involved and the danger of degenerating into a mere student of literature in the effort to make a complete catalogue; another is the fact that, if made in advance of actual need, or

\*FIG. 100.—Sample from card-catalogue. Two-thirds actual size.

†FIG. 101.—Top of large sheet used for voluminous abstracts. A red line near left-hand margin marks off a space on which summarizing catch-words or phrases are written. Breadth of sheet, 6 $\frac{7}{8}$  inches.

by some one not entirely familiar with the subject, it not infrequently happens that the statements in the paper which have been omitted from the abstract as unimportant prove in the end to be the essential ones so far as the owner of the abstract is concerned. For this reason, when they are within reach, the writer prefers to consult the original papers and to save for original work the time consumed in making long abstracts. When they are rare, frequently needed, and only to be had

by borrowing, the writer has sometimes photographed the more essential parts. In one instance a pamphlet was borrowed from Europe for this purpose.

For the exact measurement of colonies, etc., a strip of plate glass 35 cm. long and ruled into 350 mm. spaces may be had from Carl Zeiss, and will be found very convenient (fig. 102).

Steel rules of any size and of very excellent workmanship, graduated according to the English or the metric system in any degree of fineness, may be had from the L. S. Starrett Company, Athol, Mass. Two of these rules much used by the writer are, respectively, 12 inches and 30 centimeters long. They are one inch wide and about three sixty-fourths of an inch thick. They are graduated on both sides, the metric rule into centimeters, millimeters, and one-half millimeters, and the English into inches, halves, quarters, eighths, sixteenths, thirty-seconds, and sixty-fourths.

Stage micrometers made by Zeiss are recommended for the finer measurements. These have 1 millimeter divided into tenths, twentieths, and one-hundredths very accurately. All the magnifications of microscopic



Fig. 102.\*

objects figured in this book are recorded in terms of such a micrometer. After the drawing has been made it is customary to substitute for the section-slide this stage micrometer and throw the image of some portion of the ruled scale on the paper

\*FIG. 102.—Green cucumber soft-rotted by *Bacillus aroideae*. Contents emptied out and skin filled with water and so photographed, 3 days from date of inoculation, which was by means of a few needle-pricks. The fruit was kept at about 25° C. The black bands are pencil marks on the millimeter rule placed inside. The numerous small dark spots are denser bits of tissue which did not wash free on rinsing out the sack with water. At the left drops of water may be seen oozing through the skin and falling. Photograph, nearly natural size, by Townsend.

where it is drawn, taking care, of course, in case of high magnification, to start one cross line from the outside and the other from the inside of the image of the lines. This method of recording magnifications is urged on all. It takes but a moment, does away with troublesome computations, and enables anyone at any time to determine just what was the magnification. The magnification is determined, of course, by dividing the apparent size by the actual portion of the scale shown. For

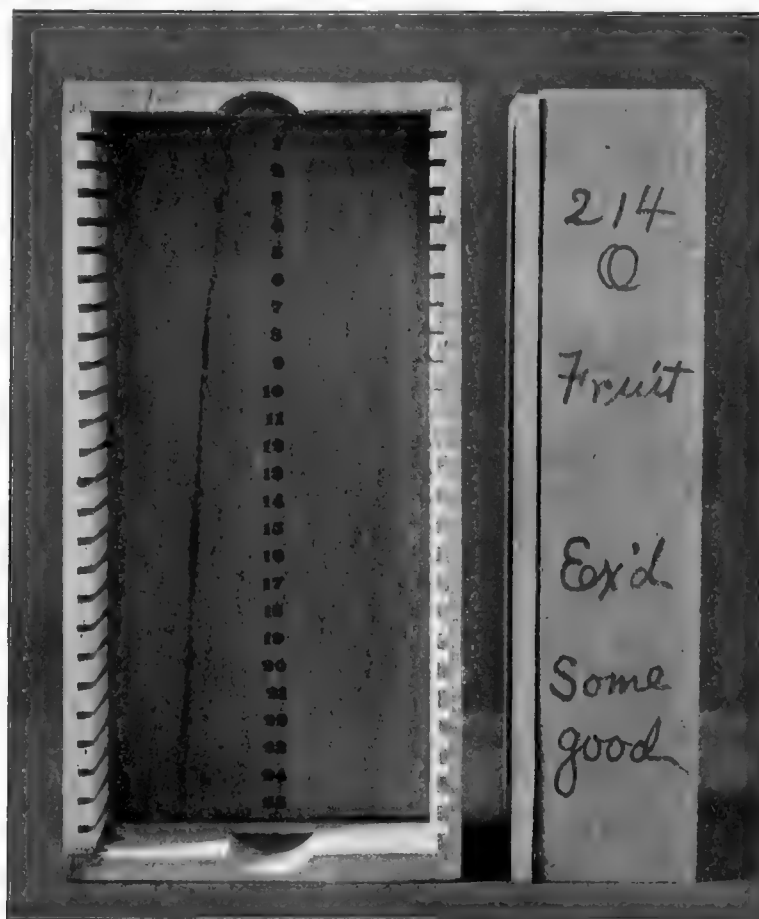


Fig. 103.\*

example, if the scale drawn on the paper is 10 mm. long and represents 0.01 mm. of the actual micrometer scale, then the magnification is  $\times 1000$ ; if it represents the entire millimeter of the micrometer scale, the magnification is  $\times 10$ .

For fine weighings, Christian Becker's balances are very satisfactory.

\*FIG. 103.—Pillsbury slide-boxes empty and full, made by Bausch & Lomb, Rochester, N. Y. These boxes are simple, inexpensive, and satisfactory, especially for serial sections.



## THE MAKING OF COLLECTIONS.

A good, representative collection of diseased material is a prime necessity in every pathological laboratory. This grows into completeness only with the lapse of much time and the aid of many hands. It should include photographs, drawings, paintings, dried material, representative specimens preserved in strong alcohol, and serial sections properly stained and mounted in Canada balsam or Dammar balsam, which must not be dissolved in chloroform, since this gradually removes the stain. With the accumulation of much material, some sort of classification becomes im-

perative. At present the writer keeps the material designed for sections in 95 per cent alcohol, arranged in as many groups as there are parasites involved. Each jar of material finally receives the same number as the paraffin block from which sections are cut. This material must be examined at least once a year to see that the alcohol has not evaporated, especially if corks are used. Only the best velvet corks should be purchased, and as an additional precaution they should be sealed in with paraffin. The negatives are filed away in similar groups, protected by negative bags. The stained sections, mounted in balsam, are filed away in cheap wooden boxes (Pillsbury boxes), each holding 25 slides (figs. 103, 104). These are very convenient, if properly made, but some boxes of this sort lead to much vexation of spirit, the grooves being too narrow to receive any but the thin-

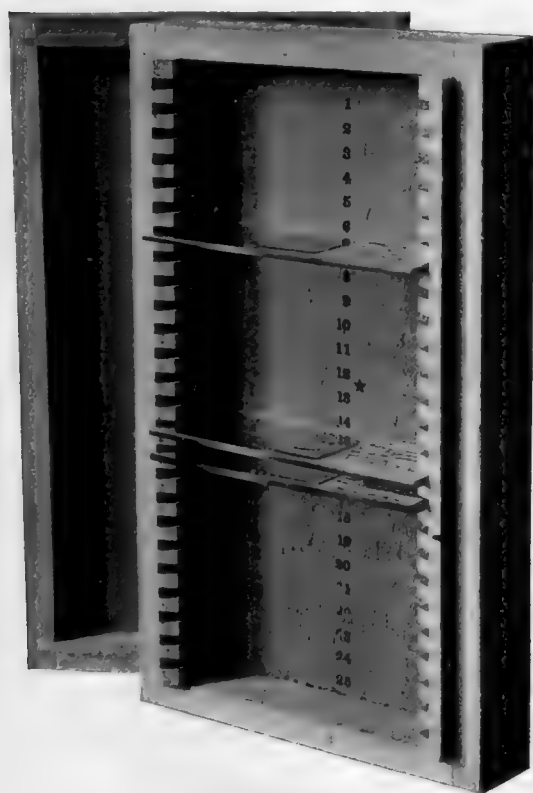


Fig. 104.\*

nest slides. Those sold in recent years by Bausch & Lomb have given no trouble. In the form shown in fig. 104 the cover remains on better and the mounted slides are easier to take out, but in drying the preparations with the cover off, these boxes tip over at the least touch. During this drying, which requires from a few days to several weeks, the slides should, of course, lie flat, not on edge.

\*FIG. 104.—Another style of slide-box. The advantages of this box are that the cover is not likely to fall off and that the slides, in case of full boxes, are withdrawn more easily. The disadvantages are that it is tipped over very easily when standing on end open, that the cover is readily mistaken for the bottom when it is closed, and that if the cover is put on upside down the writing on the edges is divided. These may also be had from Bausch & Lomb.

The writer passes material designed for sections from alcohol through chloroform (or xylol) into paraffin. Chloroform is preferred in case the infiltration is to be completed in vacuo; otherwise xylol is generally employed. A mixture of xylol and alcohol is first used, then pure xylol, after this xylol with as much paraffin as can be dissolved in it cold. The vial is then placed on top of the paraffin bath and

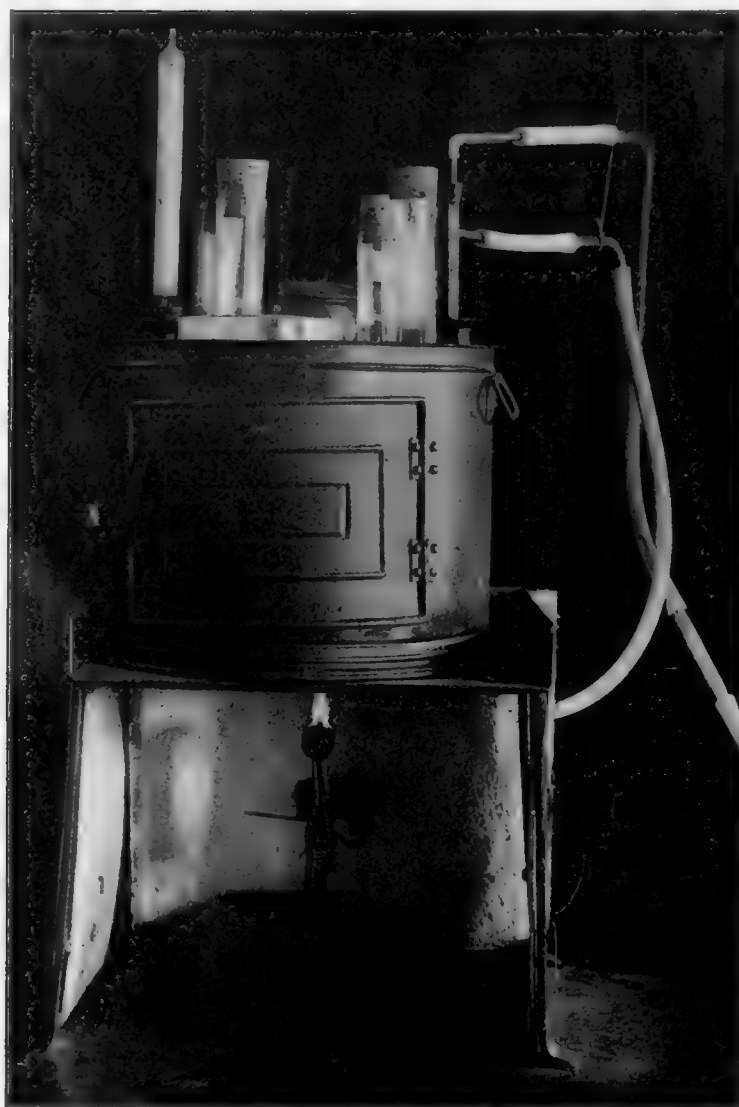


Fig. 105.\*

shaved paraffin added until it will dissolve no more at this temperature; the material is then placed inside the apparatus in pure melted paraffin, and it is finally mounted from a second dish of pure paraffin. The temperature of the paraffin bath is usually

\*FIG. 105.—A small paraffin oven much used in the writer's laboratory. The capacity of the chamber is 6 by 7 by 5 inches. The thermo-regulator is like that shown in fig. 35, but with chloroform substituted for glycerin.

kept at 59° C., and the material is subjected to this temperature only long enough to secure proper infiltration. Generally a few hours are sufficient. A small oven used for this purpose is shown in fig. 105. For large laboratories or classes of students the separate-compartment paraffin oven designed by Dr. Lillie is very convenient. Grüber's paraffin is preferred, and for the climate of Washington we use mixtures of three grades of hardness, viz, melting point 52° C., 58° C., and 60° C., increasing or decreasing the amount of the harder sorts according to the time of year. Dirty paraffin should never be used. All the stock paraffin should

be carefully protected from dust. The same remark applies still more pertinently to the sections cut on the microtome. They should be made in still air, in a clean room, and should be carefully protected from dust until stained and mounted. The paraffin-infiltration is usually a simple process unless the material contains air. The embedded material is given a serial number which is scratched on the paraffin (fig. 106), until it is fastened to the cutting block, when it is written on the latter (fig. 107). These blocks are kept as shown in fig. 108. The sections are fastened to clean slides by a *very thin* layer of Mayer's egg albumen

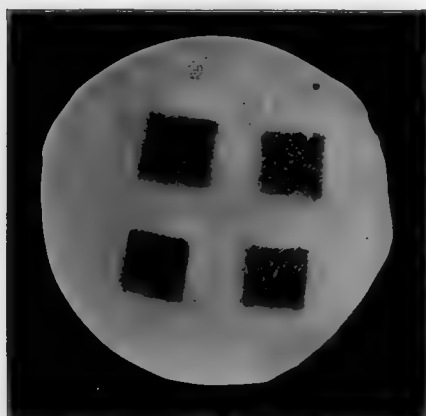


Fig. 106.\*

fixative (see Lee's *Vade Mecum*, 5th ed., p. 143), or with pure water, or preferably with 0.5 per cent gelatin water (which will not keep untreated, but may be preserved by adding 3 per cent phenol); the paraffin is removed (after cautious melting) by exposure to turpentine or xylol, alcohol is then substituted, and thereafter graded mixtures of alcohol and water down to alcohol containing 50 or 60 per cent of water, followed by the stain. Water is then removed by passing through graded alcohols into absolute alcohol; xylol or bergamot oil is substituted for the alcohol, and the section is finally mounted in balsam. Coplin's staining jar is preferred (figs. 109, 110). A series of staining jars, ready for use, is shown in fig. 111. The section properly fastened to the slide, and dry, is started in at the left after melting the paraffin with gentle heat, and is taken out at the right ready for mounting in balsam.† In this series of jars the gradations are as follows, beginning



Fig. 107.†

\*Fig. 106.—Infiltrated tissues embedded in paraffin in a watch-glass and now ready to cut out and mount on blocks for the machine.

†Fig. 107.—Infiltrated material embedded in paraffin and mounted on a pine block ready to cut on the microtome. Actual size.

‡Sections designed for photo-micrographic work must not only be cut in clean air, but mounted in *absolutely clean balsam*. So much trouble has been experienced in finding such dissolved balsam on the market that the writer now makes his own. The dry balsam is heated in an oven until all easily volatile products are driven off and it becomes brittle. It is then dissolved in xylol and filtered under a bell jar to exclude dust. The filtering usually requires several days.

at the left: Xylol, second xylol, xylol one-third absolute alcohol two-thirds, 95 per cent alcohol, 75 per cent alcohol, 55 per cent alcohol, 40 per cent alcohol, carbol-fuchsin, 40 per cent alcohol, second 40 per cent alcohol, 55 per cent alcohol, 65 per cent alcohol, 75 per cent alcohol, 95 per cent alcohol, absolute alcohol, second absolute alcohol, xylol, second xylol. From this last jar the material is mounted in balsam. Turpentine may be substituted for xylol in jars 1 and 2. After the paraffin is fully removed, the slides are passed rapidly from jar to jar (a minute or two

in each being generally sufficient) until the stain is reached. After remaining in the stain the proper length of time (usually three to ten minutes, but sometimes much longer) the slides usually are allowed to remain in the 40 per cent alcohols for a number of minutes, with frequent inspection. When they appear to be properly bleached (rather pale) they are passed rapidly through the remaining jars until they reach the xylol, in which they may remain for some time without injury, if they can not be mounted immediately, but they must not be allowed to stand for any great length of time in any of the alcohols. The secret of success lies in obtaining just the proper amount of differentiation in the 40 per cent alcohol and in not losing any of this later on. To retain the stain it

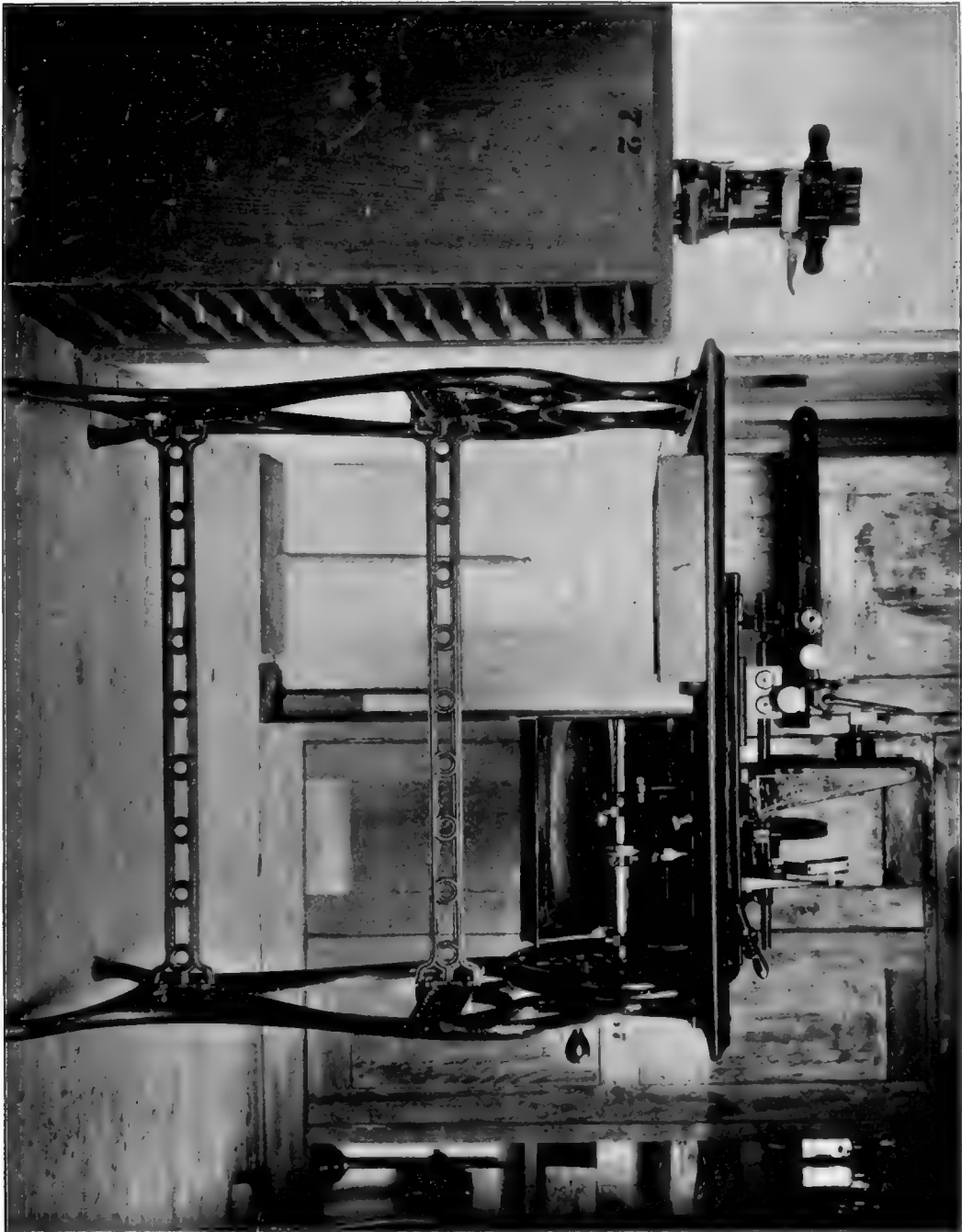


Fig. 108.\*

is necessary sometimes to omit some of the graded alcohols.

The time required for properly staining sections varies from one or two minutes to a half day or more, according to the subject and the stain employed. No general rule applicable to all cases can be given. When the material is selected for embedding, its serial number, with a full description, is entered in the record book (fig. 112).

\*FIG. 108.—One of a series of drawers divided into small compartments for holding infiltrated, embedded material, cut and uncut.



Front view of the Reinhold-Gilay microtome.

Arranged for cutting ribbon-sections from paraffin.



This book must not be lost or misplaced. The advantage of having the serial number written also on the bottle containing the stock of preserved material is very evident if a thing of this sort ever happens. The serial number is written on one edge of the slide-box, and serves to identify it (fig. 103). Some record besides a mere number should also be placed on the slide-boxes. All the slides within bear this number, *e. g.*, 256, and also a series number of their own, *i. e.*, 1 to 25. The slide-boxes are then filed away on shelves either serially or in groups, according to the parasite. Slides containing particularly good

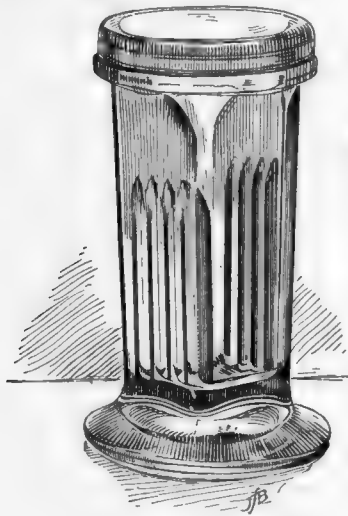


Fig. 109.\*

fields are marked X, and when the best fields are finally decided upon their location is recorded as determined on the mechanical stage. In case a dozen or more serial sections are included on one slide the extra good ones are marked X on the first examination, and the others O, as shown in fig. 113. When one of these sections has been drawn or photographed, the X is underscored or inclosed by a circle. This method enables one to keep track of any number of sections.

Free-hand sections may be made with the Torrey razor shown in fig. 114D. This is altogether the best razor the writer has used. When very dull it may be sharpened on an India oil-stone. These stones are said to be made of a mixture of carborundum and clay, baked at a high temperature. They may be

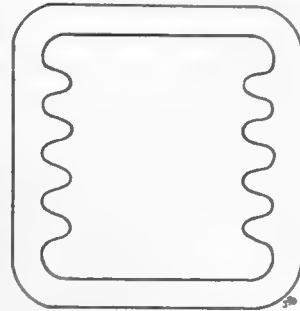


Fig. 110.†

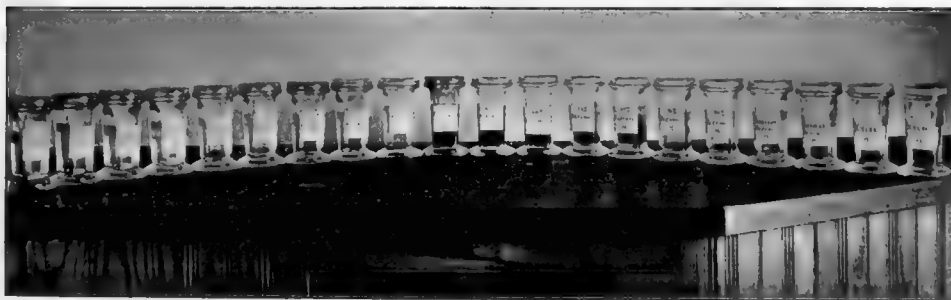


Fig. 111.‡

had of the Norton Emery Wheel Company, Worcester, Mass., in three grades of fineness, the finest being usually coarse enough for the dullest razors. The size needed is 8 by 2 by 1 inch. The finishing may be done on an Arkansas oil-stone, with a

\*FIG. 109.—Coplin's staining jar. About one-half actual size.

†FIG. 110.—Cross-section of Coplin's staining jar. About actual size.

‡FIG. 111.—A series of Coplin's jars filled and properly arranged for staining sections fastened to slides.

few final touches on a good leather strop. The maintenance of good edges on microtome knives is a matter of great importance and considerable difficulty, and where much material is to be cut it is very economical of time to send away such

knives to be put in order by some expert. In recent years the writer has sent all such knives to Charles Lentz & Sons, Philadelphia, with very satisfactory results. Knives suitable for serial sections are shown in fig. 114 A and C. In fig. 114 B is shown one of a set of knives not inclined to spring and well adapted to the cutting of hard material with a long slant stroke. These knives were made to order by Lentz & Sons at a cost of about \$6 each. An end-on view of all these knives is shown in fig. 114 a, b, c, d.

Many plant tissues, especially mature leaves, are full of very hard calcium oxalate crystals, and the difficulties of properly cutting such material are very great. The cutting of thin sections of bone would be quite as easy. After even a few sections the edge of the knife looks like a miniature saw and the sections themselves are badly torn, partly by the dulled knife and partly by the movement of the crystals themselves. In case of the yellow disease of the hyacinth the

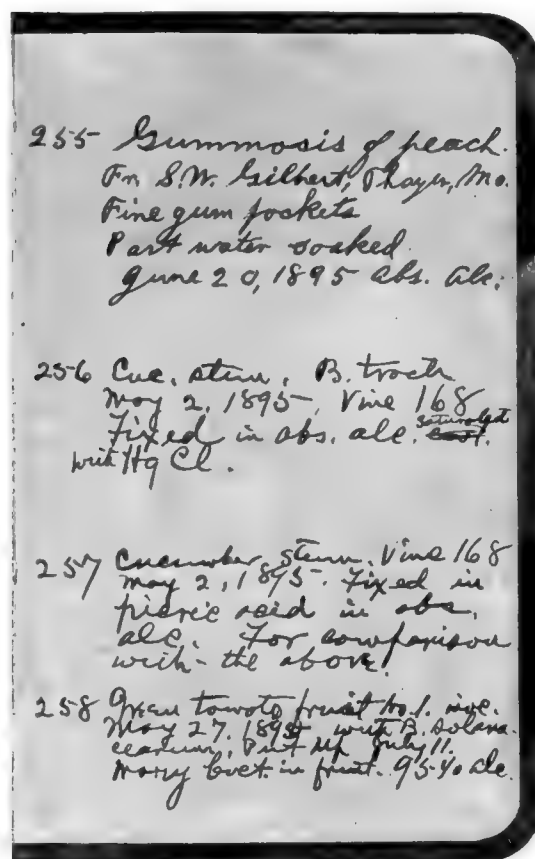


Fig. 112.\*

writer has never been able to make satisfactory thin sections, many of the soft cells being filled with bundles of very hard raphides which he has not been able to dissolve without serious injury to the tissues. In such cases thick free-hand sections are about all that can be hoped for.

Serial sections are cut on the microtome. The one shown in pl. 13 and fig. 119 leaves nothing to be desired in the way of a perfect-working durable instrument. The ribbon-carrier is above the table at the left. The knife is stationary. The block moves up and down, and the razor-carrier

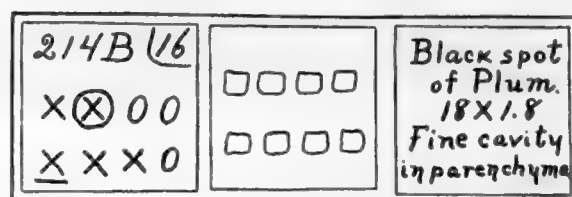


Fig. 113.†

\*FIG. 112.—A page from the paraffin record-book. The numbers on the slide-boxes (fig. 103) correspond to numbers in this book. Two-thirds actual size.

†FIG. 113.—A mounted slide of serial sections, showing manner of labeling.



moves forward at each stroke a distance governed by the set-screw of the scale ( $\frac{1}{2} \mu$  to  $40 \mu$ ). By substituting a wide knife-carrier, sections several centimeters in diameter may be cut, and by using a slanting knife, as for celloidin, very hard material may be cut. By loosening a set-screw, the razor as here shown may be turned a few degrees to right or left, and the paraffin block may also be moved through a considerable arc in any direction, it being held securely in any position by pressure of a collar-screw on a ball-and-socket joint. On 72 in plate 13 is an apparatus for truing the edges of the paraffin blocks.

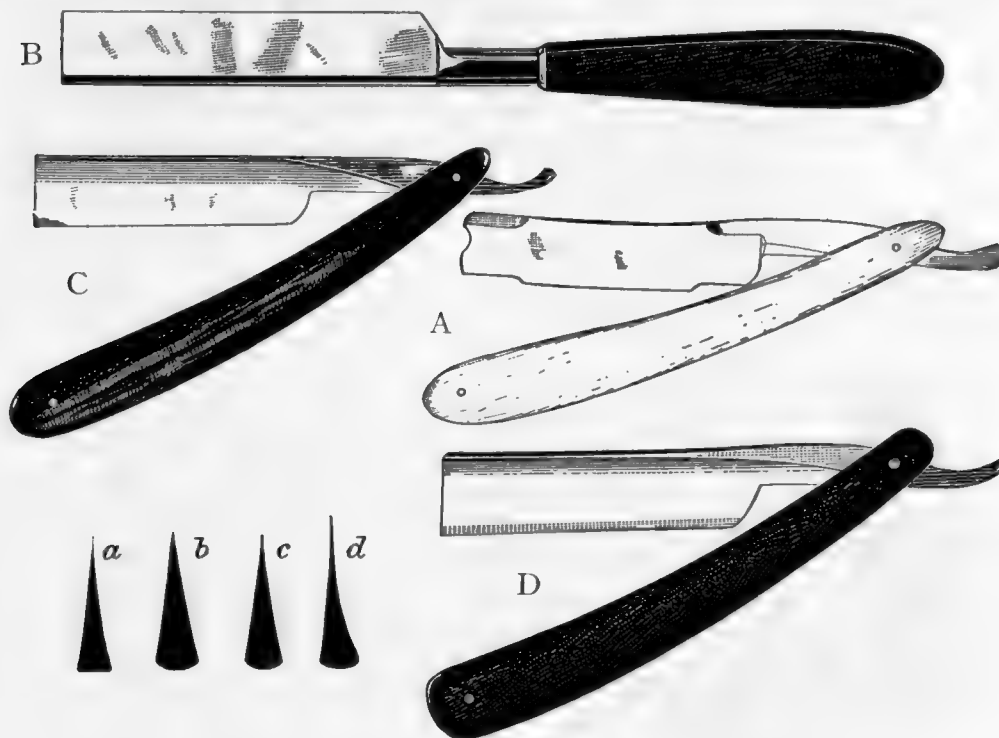


Fig. 114.\*

Collections of living bacteria are also necessary. Fortunately many may now be obtained, as needed, from Král, in Prague; but, unfortunately, they do not always correspond to their name. Others must be kept on hand, and the cultures (of some sorts) must be renewed at frequent intervals. That way which has given the writer

\*FIG. 114.—A. Knife for serial sections, furnished with the Reinhold-Giltay microtome. This is made by Joseph Rodgers & Son, Sheffield, England. One-half actual size.

B. Microtome knife made to order by Charles Lentz & Sons, Philadelphia, and found useful in cutting hard material with long slant strokes. One-half actual size. The broad wedge-shaped blade of this knife is shown in *d*.

C. Knife obtained from J. R. Torrey & Co., Worcester, Mass., and found very useful for making serial sections on the microtome. One-half actual size.

D. Torrey razor, recommended for free-hand sections. The very thin blade is flat on one face and hollow-ground on the other, as shown in *d*. It is made of the very best steel and holds an edge well. One-half actual size.

*a, b, c, d*, end views of the cutting edge of knives shown in A, B, C, D. Actual size.

least inconvenience is by storage in cool boxes (refrigerators) at temperatures of  $10^{\circ}$  to  $15^{\circ}$  C. By this method some organisms can be kept alive on agar a year without transfer, and even sensitive organisms will generally live for some months, especially

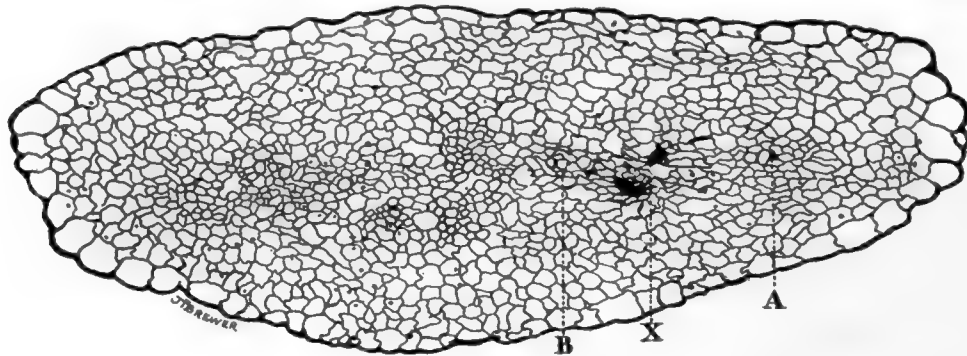


Fig. 115.\*

if planted in proper media. The writer has never made any attempt to prepare a collection of dead bacteria on culture media to serve as museum specimens, but it is possible to do so, it is said, with considerable success by following the methods described by Hauser and others (Bibliog., LII).

#### DISTILLED WATER.

All laboratories doing much work should have an abundance of distilled water, and where this is not readily obtainable in sufficient quantity and of good quality, provision should be made for it when the laboratory is constructed or when the necessity for it arises. In the construction of such a still many things must be kept in mind, if it is to work satisfactorily and yield water of the desired purity.†

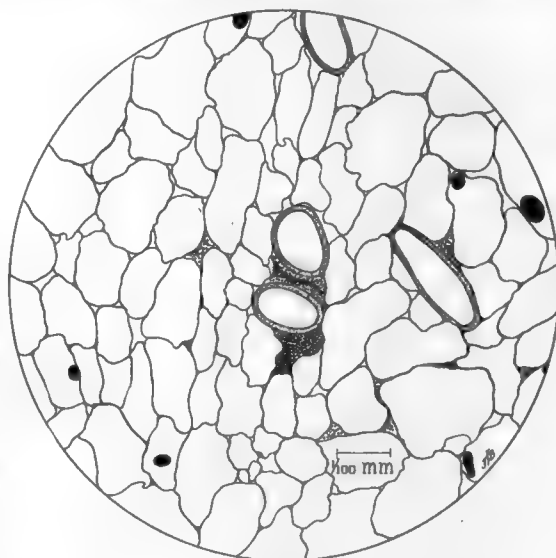


Fig. 116.†

\*FIG. 115.—Cross-section of tooth of cabbage-leaf infected by *Bacterium campestre*. Plant No. 401 sprayed with water containing an agar-culture. Bacterial occupation limited to points between A and B. At X vessels are occupied. At A and B the bacteria lie in the intercellular spaces and have not yet entered the vessels. For details of A and B, see figs. 116 and 117. This section, which is one of a series, was cut  $270\mu$  below the apex of the leaf-tooth. A few micromillimeters further down ( $370\mu$ ) all trace of the bacteria disappears. In other words, the bacteria are still confined to the leaf-tooth, and there is no cavity like that shown in fig. 76. When sprayed this leaf was extruding fluid from the water-pores. Actual length of section, slightly under 1 millimeter. Slide 331c 3. Plant sprayed December 9, 1904; slightly blackened leaf-tooth fixed in 95 per cent alcohol on December 17, 1904. Inked from a photomicrograph.

†FIG. 116.—Cross-section of leaf-tooth of cabbage infected by *Bacterium campestre*. A detail from fig. 115 at A. The bacteria have not yet entered the vessels.

‡That thing which has given the writer most trouble was an entirely unexpected difficulty, viz, a plague of tiny red house ants. These got into the reservoir in spite of all that could be done to render it tight, and, of course, spoiled the water for all delicate work.



Apparatus for Distilling Water.

- (1) Steam inflow pipe; (2) waste-steam pipe; (3) hydrant-water inflow pipe; (4) hydrant-water outflow pipe (flush) to sewer; (5) galvanized-iron boiler; (6) water gage; (7) brass top, tinned on the under side; (8) copper catch basin; (9) steam safety valve; (10) block-tin steam pipe to condenser; (11) block-tin water pipe from condenser; (12) hydrant-water pipe into condenser tank; (13) hydrant-water pipe from condenser tank; (14) flush pipe for condenser tank; (15) reservoir, capacity 80 gallons; (16) water gage; (17) overflow pipe from reservoir; (18) block-tin pipe leading to various rooms; (19) iron support.



The following description and figure of a distilled-water apparatus devised by the author for use in the Laboratory of Plant Pathology, United States Department of Agriculture, may be of interest, therefore, to some. The apparatus consists of a galvanized-iron boiler similar to those used in kitchen ranges. It is 18 inches in diameter and about 5 feet high. The top is sawed off and to it is bolted a stout iron ring with a flange, on which rests a  $\frac{1}{4}$ -inch brass cover. In the lower half of this boiler is a coil of 52 feet of inch copper pipe, the upper end bent downward and *securely fastened* in the bottom of the boiler to a steam pipe (1 inch) connected with a  $1\frac{1}{2}$ -inch steam pipe leading to the ordinary steam boiler in the engine room in the basement; the lower end connected with an iron steam pipe (1 inch) leading to a steam trap (Mark traps are said to be the best). Around this copper steam

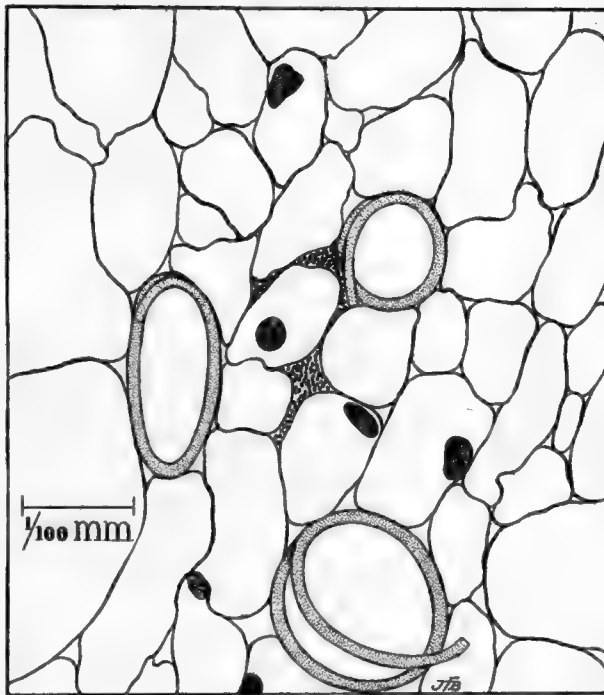


Fig. 117.\*

pipe, which is of course tin-plated, stands the river water which is to be converted into steam by contact with the hot pipe. This hydrant water is kept always at about the same level (level of fig. 5 in plate 14), by means of a tinned-copper ball float (automatic cut-off) which closes the mouth of the inflow pipe when the water rises beyond a certain point. The upper part of the cylinder is a steam chamber under very moderate pressure (0 to  $\frac{1}{2}$  pound, rarely more). The excess of pressure is dissipated either by escape of steam through the safety valve (9), which is not weighted, or through the coil of pipe in the condenser. The steam passes from a securely riveted tin-lined

copper catch basin (8) into a  $\frac{3}{4}$ -inch block-tin pipe (10), which is fastened to a tubular projection from the catch basin by means of a collar screw. The tubular projection from the top of the catch basin is soldered in place and also held by a flange inside the copper top, so that it can not be forced out by any attainable degree of steam pressure. The  $\frac{3}{4}$ -inch block-tin pipe passes to the room above, where it is coiled for a length of 35 feet inside a tin-lined copper tank resting on the floor. The height of the condensing tank is 18 inches and its diameter is the same. When in operation this tank is full of running water. Theoretically, this condensation tank is large enough, and it is so practically when the hydrant pressure

\*FIG. 117.—Detail from fig. 115 at B, showing an early stage of water-pore infection of cabbage. The bacteria have not yet entered the spiral vessels. The large dark bodies are nuclei.

is good, but when it is feeble or when the steam pressure is high the water becomes too hot and steam sometimes escapes into the reservoir. The water therefore must be hurried through the tank by the use of a steam pump, or else less steam must be allowed to enter the copper pipe. If the writer were to build another similar apparatus he would make the condensing tank 2 feet higher and add 10 feet to the length of the coil of tin pipe. The condensing tank is provided at the bottom with a 1-inch inflow pipe for the cold water (it should be  $1\frac{1}{2}$ -inch), and at the top

with a  $1\frac{1}{2}$ -inch outflow pipe (it should be 2-inch), for the exit of the warmed water. There is also a 1-inch flush pipe at the bottom for the occasional removal of sediment.

The size of the outflow pipe, which must be somewhat larger than the inflow pipe, prevents any possibility of clogging and overflow. All the metal parts which come into contact with the distilled water are tinned or nickel-plated. Connected with the lower end of the block-tin coil (by tin solder, which must not contain lead or zinc) is a smaller ( $\frac{1}{2}$ -inch) block-tin pipe (11), which leads the distilled water into (15) the storage tank ( $\frac{3}{4}$ -inch pipe would be better, and without any joint). The reservoir in this case is a white-enameled bath-tub, on the top of which is clamped down a cover of thin sheet copper ( $\frac{1}{60}$ -inch), the inner face of which has been carefully tinned. Plate glass ground to fit would be better, and the tub itself is likely to be discarded in the near future, *i. e.*, when some more satisfactory storage tank can be found. The problem of the proper storage of distilled water in quantity is the hardest one, the solvent power of the water is so great. From the bottom of

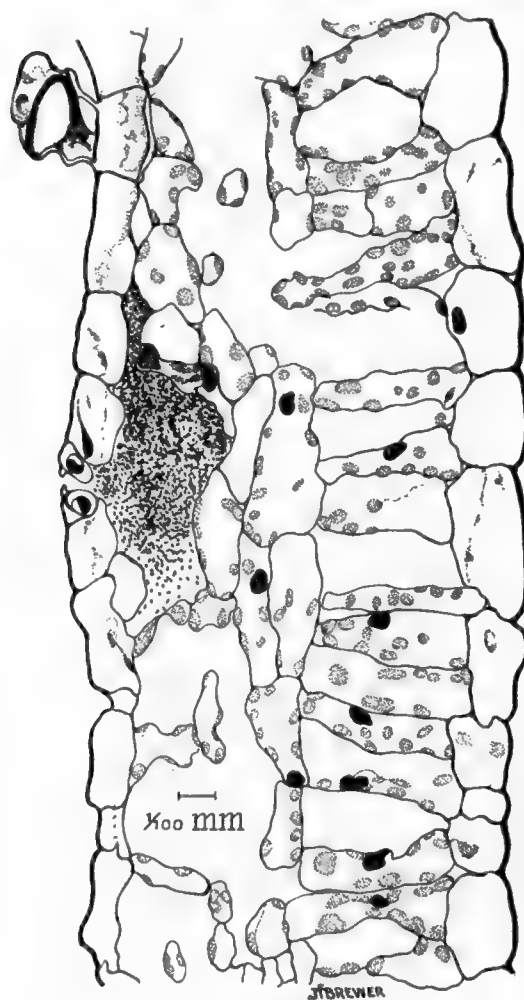


Fig. 118.\*

this bath-tub several hundred feet of  $\frac{1}{2}$ -inch block-tin piping lead to various rooms in the building. In addition to the terminal faucets there is a general cut-off just above 18, which is necessary in case of an accident to any faucet or part of the piping. There is also an overflow pipe (17), which does not enter the sewer, but

\*FIG. 118.—Early stage of stomatal infection in angular leaf-spot of Rivers cotton. Hothouse infection produced by spraying *Bacterium malvacearum* upon the surface of the leaves. For a much later stage see fig. 80.

ends free in the laboratory about 1 foot above a deep sink. The sides and top of the boiler, the copper catch basin, and the  $\frac{3}{4}$ -inch block-tin pipe leading to the condenser are all coated with 3 inches of best non-conducting magnesia covering. The catch basin, designed to hold back solid particles carried up with the steam, is 9 by 12 inches and is made of  $\frac{1}{16}$ -inch copper, securely riveted and soldered with tin solder. It is bolted down to the flat brass top and a steam-tight connection

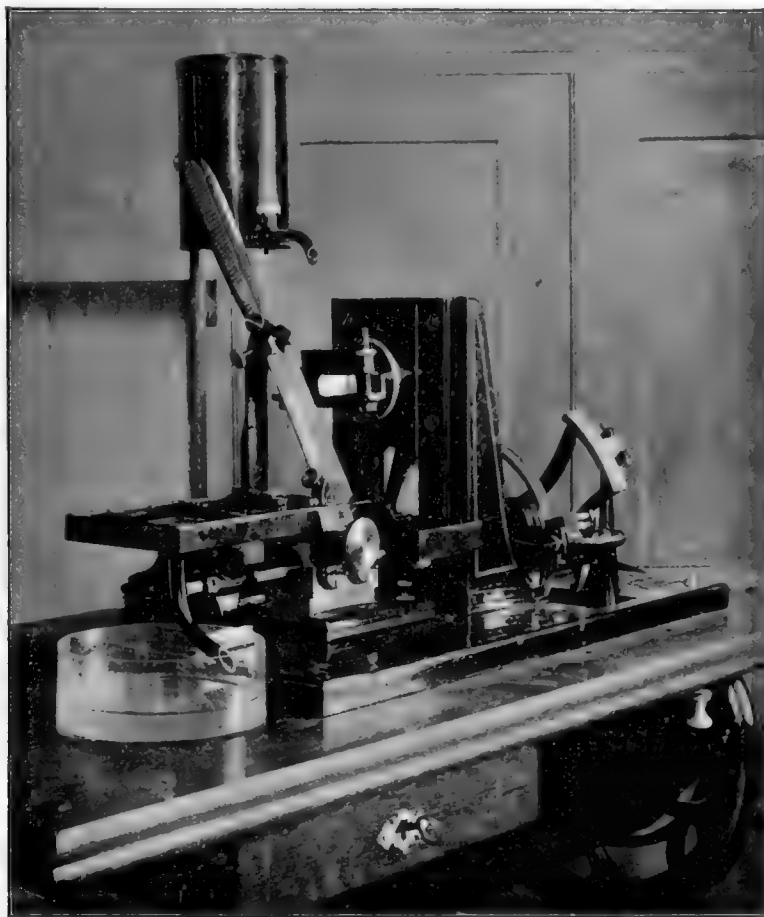


Fig. 119.\*

is secured by means of a red rubber gasket. The heavy brass top (7) is tinned on the inner surface and is bolted securely to the iron flange on the top of the boiler by means of 18 screw-bolts. The junction is made steam-tight by means of a corrugated

\*FIG. 119.—The Reinhold-Giltay microtome arranged for cutting celloidin or very hard paraffin sections. The machine is very solidly and accurately constructed out of the best materials, and, in addition, provision is made by means of set-screws for compensating the wear due to long use. The device governing the thickness of the sections is especially ingenious. This particular machine has been in constant use by various persons for over four years, and nothing has been paid out for repairs. With good use it ought to last a lifetime. About one-fifth actual size.

tinned-copper gasket. The steam which runs the apparatus is brought to the laboratory floor through a 1½-inch pipe, in which (in the engine room) there is a steam gage registering up to 150 pounds, and a reducing valve set at 55 pounds. This very considerably lessens the steam pressure in the copper coil, moderates the violence of the ebullition, and makes the apparatus perfectly safe. The hydrant-water outflow pipe (flush) to the sewer, for occasionally washing out accumulated mud (4) passes from the bottom of the boiler immediately above fig. 19. Gate-valves are used. All brass and copper parts in contact with the steam are tinned; all metal parts in contact with the distilled water are tin, tinned, or nickel-plated.

With 60 pounds steam pressure in the engine-room boiler, 40 pounds pressure at the reducing valve, 35 pounds pressure in the pipe at the laboratory floor near where it enters the still, and one-half pound pressure or less in the steam chamber above the coil of copper pipe, the capacity of this still is 60 liters (16 gallons) per hour.

The apparatus must be built very substantially in all parts, so as to withstand at least twice as much steam pressure as any part of it will be subjected to, *e. g.*, 160 pounds in the iron pipes and in the copper coil and its attachments, and at least 20 pounds in the catch basin, and other parts subject to steam generated in the still. A steam gage, in addition to the one in the engine-room, shows the pressure in the coils, and another the pressure in the steam chamber above the coils. They are not shown in the plate, as they were put on after that was made. The former is attached to the steam supply pipe near the floor, and the latter to an arm of the safety-valve pipe. The boiler should be taken down and the parts retinned once a year, or at least once in two years.

If a much greater quantity of water is needed the block-tin condensation coil should be lengthened to 60 feet, the diameter of the inflow pipe of the condenser should be increased to 2 inches, and the outflow pipe to 2½ inches, and the cubic contents of the condenser tank should be quadrupled. The capacity of the bath-tub (or other receptacle), for a large laboratory should be at least 500 liters, and might well be 1,000 liters.

The above apparatus has been in use for two years. It works very smoothly and satisfactorily when the proper amount of steam is let into the coil of copper pipe, which ordinarily should not be nearly the whole amount available. The inflow of steam is governed by the valve a few inches below fig. 1 in plate 14. When too much steam enters the coil, the pressure in the steam chamber above it rises to five pounds or more, hot water is forced back through the feed pipe (3) into the neighboring pipe which furnishes cold water to the condenser (12), and steam instead of distilled water is furnished to the water tank. This is at once obviated by cutting off part of the steam inflow and moderating the force of the boiling. It might also be obviated by reducing the length of the arm of the safety valve (9), which in any event should not be weighted.

Sufficient water for small quantities of culture-media and pure enough for most purposes may be obtained from the simple glass still shown in fig. 82 by one distillation. Water of a high degree of purity may be obtained by two distillations, adding 0.5 gram to 1 gram of potassium permanganate per liter of water before the



first distillation, and 5 grams of c. p. sulphuric acid per liter before the second distillation. The flasks in which such water is collected or stored should be of resistant (non-soluble) glass and absolutely clean to begin with. With use such flasks or bottles become more valuable and should not be employed for other purposes.

The solubility of glassware is best tested by determining from time to time the degree of electrical conductivity of pure water stored in it. The specific resistance of pure water stored for a week in such tubes, flasks, or bottles should not fall below 250,000 ohms. The specific electrical resistance is determined upon 1 cubic centimeter of water exposed between electrodes having an area of 1 square centimeter, and is read by means of a special Wheatstone bridge. Distilled water redistilled with chromic-acid cleaning mixture, and afterwards with alkaline potassium permanganate (method used by the Physical Laboratory in the Bureau of Soils) gives a resistance of 700,000 ohms.

The following determinations made by the Physical Laboratory of the Bureau of Soils show the diverse behavior of two lots of clean test-tubes recently purchased as non-soluble glass by the Laboratory of Plant Pathology.

Kind of tube.	Time of exposure, in days.	Specific resistance, in ohms.
Resistant test-tubes, (R) from Greiner & Friedrichs.....	10	220,000
Do., 2d test.....	11	219,000
Tubes received from the School Sup- ply Co .....	10	41,400
Do., 2d test.....	11	34,000

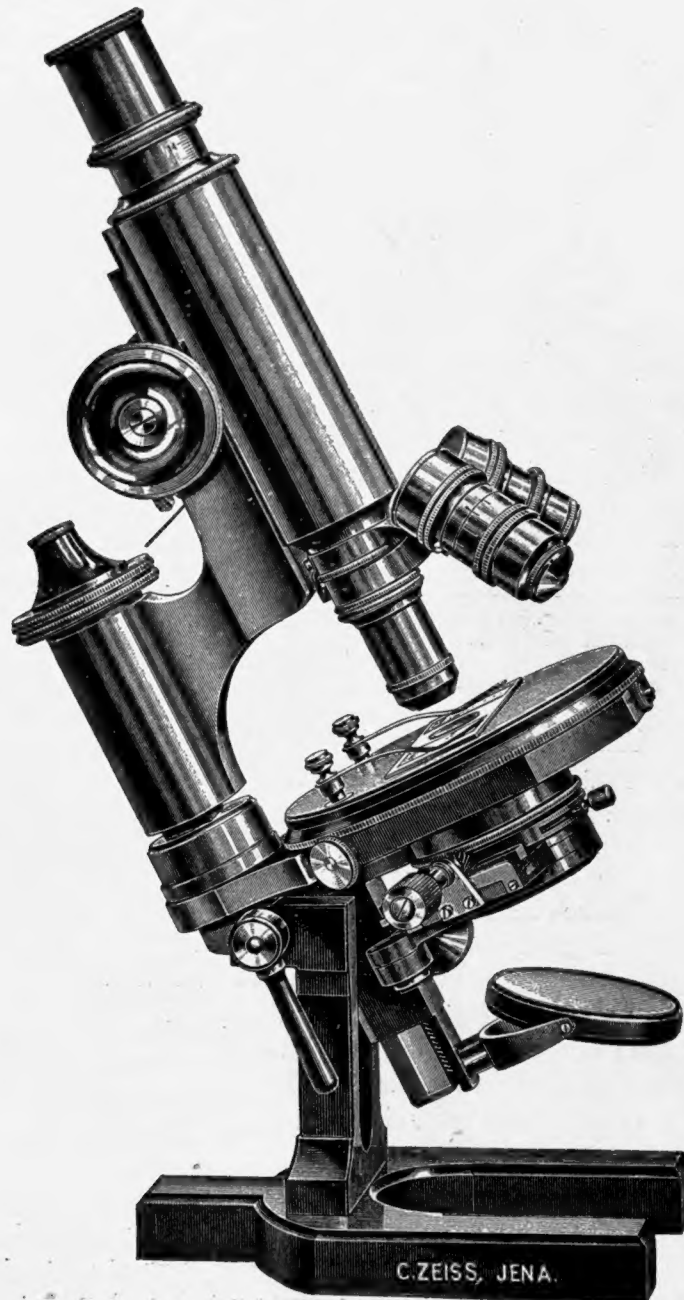
The twice-distilled water used was taken from a Jena flask and its initial specific resistance was 240,000 ohms.

#### MICROSCOPES.

Microscopes of a much better grade are required for bacteriological investigations than for ordinary histological work. The writer has for many years employed those made by Carl Zeiss, of Jena, as, on the whole, most serviceable. Good microscopes are also made by E. Leitz, of Wetzlar, and recently by the Spencer Lens Company, of Buffalo, N. Y. The Zeiss stand shown in plate 15 does very well for all ordinary work, but is not well adapted for the making of photomicrographs or for recording the exact location of particular spots in the section. The latter difficulty may, however, be overcome by means of a removable slide-carrier attached to the stage. The stand may also be used with the small upright photomicrographic outfit shown in fig. 24 when the lens does not require a microscope having a wide tube. This microscope has a half-mechanical stage, an excellent fine adjustment, and good substage apparatus. It is thoroughly well made and very durable. One in the writer's laboratory has been in use for twelve years. The lacquer has disappeared in places and it is no longer attractive to look at, but it has required no serious repairs during this time and is still serviceable.

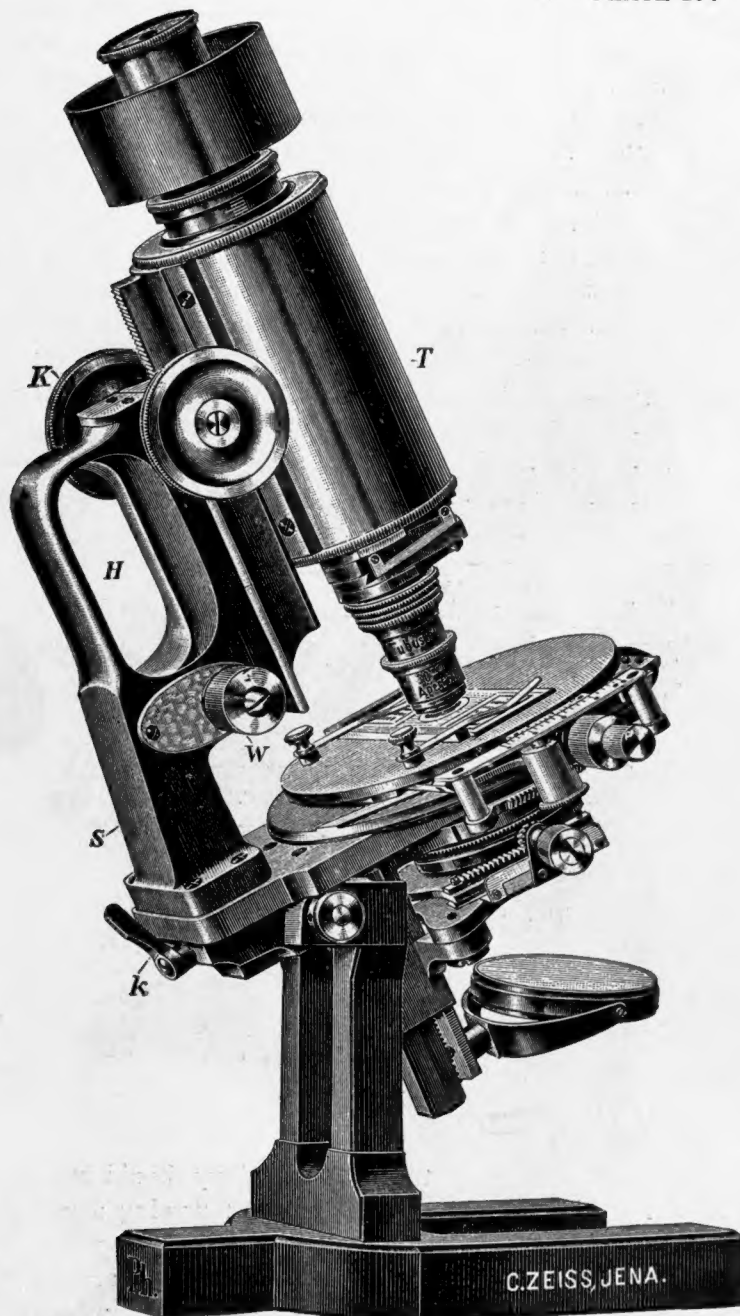
For photomicrographic work and also for recording the exact location of desirable fields in a section, the writer uses the large photomicrographic stand shown in plate 16. This is provided with a specially wide barrel, a fine adjustment of very

PLATE 15.



Zeiss microscope stand II<sup>a</sup>.

This form of microscope and that represented on plate 16 are the two patterns used principally in the Laboratory of Plant Pathology, U. S. Department of Agriculture. The objectives are apochromatic, and have proved very serviceable. In carrying do not grasp by any part above the level of the stage, as this brings an undue weight upon the fine adjustment. Seize by the base.



Zeiss photomicrographic stand I<sup>c</sup>.

The barrel "T" is of greater diameter than in stand IIa. The fine adjustment is at "W" and no weight rests on it in lifting the instrument by the handle "H." The set screw "K" locks the upper part of the instrument at any angle. The objective is set in place by means of a very convenient slide carrier. The fine adjustment screw has an extremely slow movement; and the vernier screws are on the same axis (a great convenience). The stage rotates and may be locked at the desired place by means of a set screw. For the substage arrangement see figure 120.

slow movement, a swing-out condenser (fig. 120), two substage iris diaphragms, and various other conveniences. For example, the screw-heads, determining the cross and sidewise movement of the section, are on the same axis and may be reached and moved without changing the position of one's arm.

The apochromatic objectives are the only ones recommended for bacteriological work. They cost more than achromatic objectives, but expense is a minor consideration. In hot, moist climates the older apochromatic objectives of Zeiss frequently became clouded, but those made in recent years have given the writer no trouble in the latitude of Washington. They yield sharp images even with high eye-pieces. Of course, compensating oculars must be used with the apochromatic objectives. It is desirable to have the whole series of objectives and eye-pieces, but if one is limited for means, very good work can be done with two objectives and three oculars, viz, objectives 16 mm. and 3 mm. 1.40 n. a., and compensating oculars 4, 6, and 12.

The newer forms of the Abbe camera furnished by Zeiss (fig. 121) leave little to be desired in the way of a drawing camera.

#### PHOTOGRAPHY AND PHOTO-MICROGRAPHY.

For permanent records nothing equals photography. It constitutes, therefore, a very important special part of laboratory work, and every student of pathology should make a knowledge of this subject part of his education. Some of the following suggestions will be useful to beginners.

The Zeiss Double-Protar lenses, series VIIa, are the best all round photographic lenses made by that firm, and are excelled by none made by any firm. The back or front lens is usually as good as the combination. Excellent photographic lenses are also made by Voigtlaender and by Goerz. Zeiss photographic lenses may be

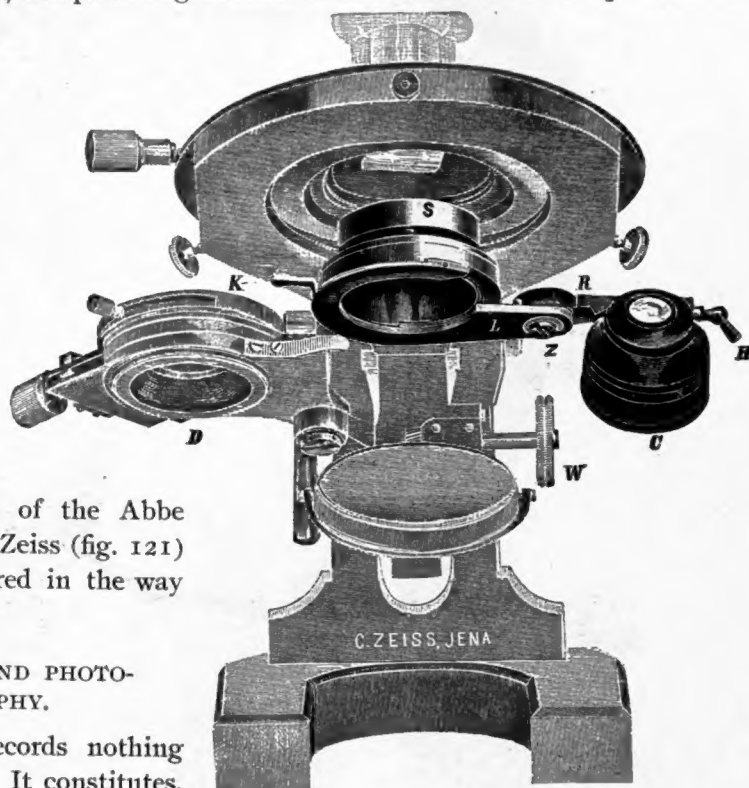


Fig. 120.\*

\*FIG. 120.—Swing-out condenser and other substage arrangements on Zeiss photomicrographic stand, No. 1c. There is an iris diaphragm in D, and a second one in S, which is for use when the condenser is thrown out as shown in this figure. D swings under when C is thrown into place. W racks the entire substage up or down.

obtained from Bausch & Lomb, who are under contract to manufacture them according to the Zeiss formulæ. In buying a photographic outfit it is economy to get one of the high-priced lenses. It is frequently stated, by those who do not know, that "just as good results" can be obtained with cheap lenses, but one may easily satisfy himself that such is not the case by photographing buildings on a

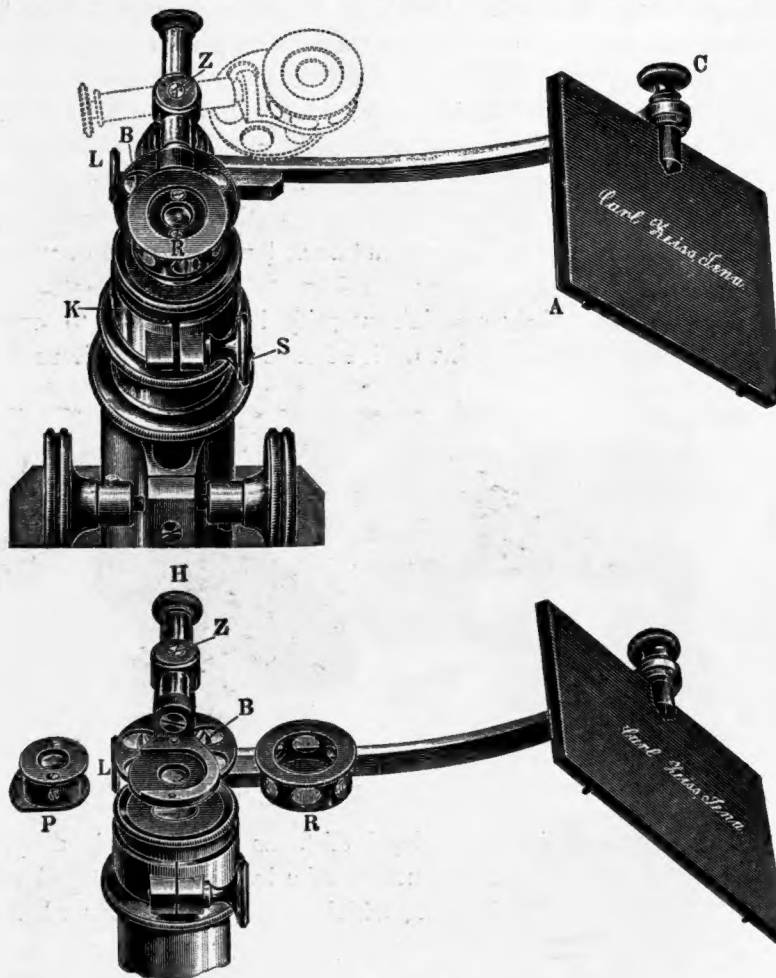


Fig. 121.\*

street or any object having many vertical parallel lines and other lines crossing at right angles. The pictures made by the cheap lenses generally show serious distortions. In buying a lens one should know in advance exactly what he wishes to do with it, otherwise he may be greatly disappointed. If he wishes to photograph only

\*FIG. 121.—Newer form of Zeiss-Abbe drawing camera. The camera is clamped at K by means of S. The prism within R is centered over the eye-piece by screw movements of L and Z. When not in use the prism is swung to the right, as indicated by the dotted lines. The mirror A throws down the prismatic image to the drawing paper. The amount of light is governed by the substage iris-diaphragm and by rotating B and R, which contain smoky glasses of graded densities. P is an extra prism. The image on the paper will also be clearer if it is placed in shadow by means of a screen of some sort.